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SCHOOL OF APPLIED SCIENCES
PROF. SHILPA B.R. CONVENER, HEAD, DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF APPLIED SCIENCES

Department of Biochemistry and Biotechnology School of Applied Sciences,
REVA University, Rukmini Knowledge Park, Yelahanka,
Kattigenahalli, Bengaluru, Karnataka, India. www.reva.edu.in

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IMPACT OF SPERM DNA FRAGMENTATION INDEX ON THE CLINICAL OUTCOME IN ASSISTED REPRODUCTION

Aamir Javed[1], Muralidhar TS[2],Manjula KR[1*]
[1]Dept Of Biotechnology, REVA University, Rukmani Knowledge Park,Kattigenahalli, Yelahanka, Bangalore-560064.
[2]Post Graduate-Department of Biotechnology, DSU, S.M. Hills KS Layout, Bangalore-560078.

ABSTRACT: To study the impact of sperm DNA fragmentation index (DFI) on the clinical results in assisted reproduction via Intracytoplasmic sperm injection (ICSI) and In-vitro fertilization (IVF).Infertile couples were enrolled in the Base fertility medical science pvt ltd from July 2014 to December 2017. All the enlisted 290 couples were separated into different categories based on their type of assisted reproductive procedure (200) for ICSI and (90) for IVF. DFI of male partner were divided in three groups, were recognized by sperm chromatin structure examination (SCSA). Group A (DFI<10%), Group B (10 %≤DFI<15%) and Group C (15%≤DFI<25%).Then, the semen samples were collected on the day of oocytes retrieval of female partner and performed the routine semen analysis, washing and DFI testing. The rates of fertilization, cleavage, good quality embryos formation, embryos implantation and clinical pregnancy was judged and compared among three DFI groups. During the normal stimulation cycle, when (DFI≤15%,) there was no statistical difference between the IVF and ICSI in the rates of fertilization, embryo cleavage, good quality embryos, embryos implantation and clinical pregnancy (P>0.05).However when the DFI percentage increases (15%≤DFI<25%), the rates of fertilization and clinical pregnancy rates in ICSI were significantly higher than those in conventional IVF (P<0.05), but surprisingly there was also no noteworthy difference between good quality in IVF and ICSI (P>0.05).The increase of DFI level can lessen the sperm motility as well as influence the clinical results of IVF and ICSI. While the results of IVF and ICSI are statistically similar when DFI level is less 15%, the overall impact of ICSI is better when DFI surpasses 15%.

KEYWORDS: Sperm DNA fragmentation, in vitro fertilization, intracytoplasmic sperm injection.

***Corresponding Author:** Manjula KR Ph.D.
[1]Dept Of Biotechnology, REVA University, Rukmani Knowledge Park,Kattigenahalli, Yelahanka, Bangalore-560064.Email: manjulakr@reva.edu.in

1. INTRODUCTION

Sperm DNA fragmentation (SDF) is also called spermDNAdamage.It has been affirmed that index of SDF is fundamentally higher in semen of infertile patients. Consequently, the integrity of sperm DNA fragmentation isn't just the viable assessment to assess the quality of sperm, yet additionally the clinical and biological marker to mirror the male fertility [1-3]. Studies have demonstrated that an abnormal state of SDF may influence the normal fertility, the natural fertilization, (for example, intrauterine insemination), and the curative effect of assisted reproductive technology (ART) [4-5]. Consequently, these outcomes can coordinate the use of ART. These days, this issue still has contentions, which may be caused by the diverse level of SDF, however it is a vital issue when IVF and ICSI are broadly connected in the male infertility of sperm irregularity [6]. This study investigated the relationship between's the different levels of SDF and the clinical results of in vitrofertilization (IVF) and intracytoplasmic sperm injection(ICSI).

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2. MATERIALS AND METHODS

2.1 Clinical Methods

Two hundred ninety couples, who were treated with IVF and ICSI, ART stimulation cycles at Base fertility medical science pvt ltd, Bangalore, India from July 2014 to December 2017. Rawsemensamplefrom290subjectswascollectedinassistedreproductive centre facilities. Tests were gathered by antegrade technique having the sexual abstinence of 2-5 days and were analyzed by World health organization(WHO)criteria(2010) [7].Asmallamountofthesemen fromeach subject test from each enrolled identifier was frozen in liquid nitrogen for future use. An informed lawful consent (ICMR BASED) was procured from all patients and benefactors. Inclusion criterion: Couples with normal karyotype and female partner with normal ovarian function whose infertility was purely due to tubal factor and agedlessthan35yearsold. Exclusion criterion: Male partner with severe oligoasthenospermia; female spouses who had sufferedpolycystic ovary syndrome (PCOS),adenomyosis, endometriosis and hydrosalpinx[8-9].

2.2 Instrument And Reagent

Computer-assisted sperm analysis (CASA-RUN-A000) from Micro Optics Company was adopted. BD FACS Caliber was employed for SDF detection. DFI reagentwas purchased from SAR health line and Halo sperm Ltd.

2.3 SDF Detection

Sperm chromatin structure assay (SCSA) and flow cytometry were utilized to identify SDF. The identification guideline and operational approach were as per the techniques proposed by Jurewicz et al. [10]. After acid treatment of semen, the sperm DNA which had structural damage which will further unfold the double stranded to single stranded loop which combines with acridine to produce a red fluorescence. While the normal or unfolded sperm DNA still kept a flawless twofold stranded structure, it would sparkle green fluorescence in the wake of joining with acridine orange and exhibiting a type of monomer. Subsequently, BD stream cytometry was utilized to recognize the flag of fluorescence. Having sperm denatured and recolored, if the extent of red fluorescence expanded, it could be inferred that the damage degree of sperm integrity. The DNA fragmentation index (DFI) was determined by CASA-RUN-A000. The equation was as per the following: DFI estimation of single sperm = red/(red + green) *100%. It corresponds to the ratio of denatured sperm DNA in the total DNA. DFIestimationofspermgroupwasexpressedasthe extent of ultra-group sperm, representing the proportion of damaged sperm in absolute sperm. It was vital to take note of that no less than 5000 sperm ought to be tried in each example and recognized consistently at least 2times [11].

2.4 IVF and ICSI Stimulation Treatment

The standard luteal long down-regulation protocol (LP) or the short flare-up protocol (SP) (24) was employed in every stimulation cycles [12]. For a short time, with the LP a 0.5–0.9 mg depot of gonadotropin-releasing hormone (GnRH) agonist (Diphereline, 3.75 mg; Ipsen Pharma Biotech) was administered in the midluteal phase of the earlier menstrual cycle. Ovarian stimulation with gonadotropin (Gonal-F;EMDSerono) was commenced after 14 days (typically on cycle day 3–9) from the introduction of Diphereline when pituitary down-regulation was accomplished [13]. Down regulation was confirmed by an endometrial lining of 4.5 mm or thinner, a serum luteinizing hormone (LH) level <5 U/L and a serum estradiol <50 pg/L. When SP was employed, the GnRH agonist (Decapetyl, 0.1 mg; Ferring GmbH) was given as an everyday dose of 0.1 mg commencement cycle day 2 trailed by gonadotropin (Gonal-F; EMD Serono) starting on day 3. For both modus operandi, daily doses of 150 U or 225 U Gonal-F were specified for the first 5 to 6 days, and then altered depending upon

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follicle development and the serum estradiol level [14]. Human chorionic gonadotropin (hCG) 5,000–10,000 IU was given when at least three or four follicles had reached 17 to18 mm in diameter, and oocyte retrieval was performed 34 to 36 hours later depending upon clinician assessment. The plan for deciding the ovarian stimulation treatment was that LP was employed in women with normal ovarian reserve [15].

2.5 Statistical Analysis

SPSS17.0 statistical programming was used for statistical analysis. The analysis of data was noted as mean ± standard deviation (x ±s). The comparison between the two groups was done by independent samples t-test. The X2 test was utilized to compare the rates of two groups $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Basic state of ART cycles: IVF and ICSI

There were 90 IVF cycles and 200 ICSI cycles in 290 chosen cycles. The sperm viscosity, sperm motility and morphology of spermatozoa of IVF cycles were all higher than ICSI cycles. The span of infertility of ICSI cycles was longer than IVF cycles. Nevertheless, these variations were not statistically significant ($P>0.05$), as shown in Table 1.

Table1: Basic state of ART cycles: IVF and ICSI

Group	Stimulation cycle Number (n)	Male partner age (years)	Infertility duration (years)	Sperm concentration ($*10^{*6/}$ ml)	Normal sperm morphology (%)	Sperm motility (%)
IVF	90	31.4 ± 4.2	3.2 ± 1.6	70.66 ± 31.33	4.4 ± 1.2	70.51 ± 10.32
ICSI	290	32.5 ±3.9	3.5 ± 1.2	65.22 ± 28.12	4.0 ± 0.5	61.29 ± 5.31

Note: Data presented as mean standard deviation (*x*±s)

3.2 DFI Features Of Art Cycles

The normal estimation of DFI was (14.6±8.5) % in the IVF cycles, (17.1±11.2) % in the ICSI cycle. Subsequently, the estimation of DFI was somewhat higher in the ICSI cycles. The difference was not statistically significant ($P>0.05$, Figures 1). The two ART cycles were grouped in IVF and ICSI where sperm DNA fragmentation of group A (DFI<10%), group B (10 %< DFI<15%) and group C (15 %<DFI<25%), as shown in Tables 2 and3.

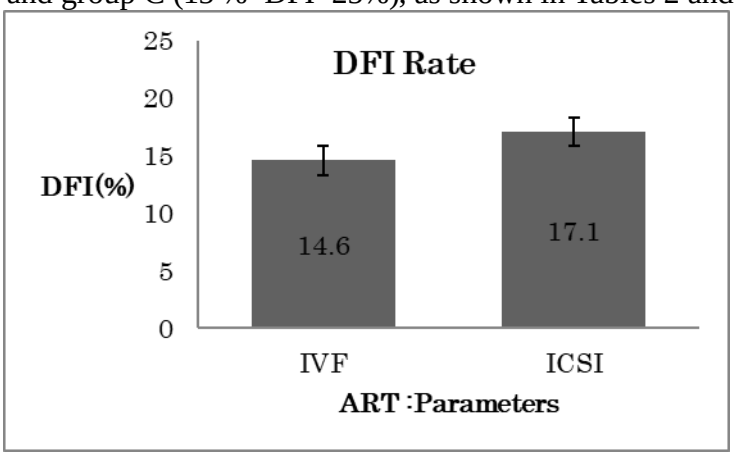

Figure 1: Estimation of DFI on IVF and ICSI group

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Table 2: Assessment of DFI values and semen parameter in IVF group

Group IVF	Number (n)	Male age (years)	Infertility (years)	Sperm concentration (*10$^{*6/}$ ml)	Normal sperm morphology (%)	Sperm Motility (%)
A	20	30.1 ± 3.2	3.5 ± 1.5	51.10 ± 25.60	4.2 ± 1.3	65.11 ± 32.21
B	28	31.3 ± 2.5	3.1 ± 1.8	59.36 ± 22.67	4.8 ± 1.8	62.88 ± 33.89
C	42	33.4 ± 1.6	2.8 ± 2.1	59.64 ± 20.36	4.1 ± 0.2	54.27 ± 22.67

Note: Data presented as mean standard deviation (*x*±s)

Table 3: Assessment of DFI values and semen parameter in ICSI group

Group ICSI	Number (n)	Male age (years)	Infertility (years)	Sperm concentration (*10$^{*6/}$ ml)	Normal sperm morphology (%)	Sperm Motility (%)
A	121	31.4 ± 5.2	4.5 ± 1.8	61.41 ± 15.60	3.2 ± 1.1	70.41 ± 22.24
B	45	33.3 ± 2.7	4.1 ± 2.9	66.31 ± 21.11	4.1 ± 2.8	68.25 ± 15.84
C	34	34.2 ± 3.6	4.8 ± 5.1	60.14 ± 10.32	3.1 ± 0.2	54.21 ± 19.66

Note: Data presented as mean standard deviation (*x*±s) and Comparison between three groups p>0.05

3.3 Evaluation of the embryo outcome with IVF and ICSI at unlike SDF levels

Theembryooutcomeswith IVF and ICSI at various SDF levels were judged among group A, B and C. The outcomes demonstrated that there was no statistical variation in the fertilization, good quality embryos formation, embryos implantation and clinical pregnancy between the two treatment strategies when the SDF level was low (DFI<15%, P>0.05), while the embryo outcomes of IVF and ICSI cycles had no noteworthy differences (DFI<15%, P>0.05), as appeared in Figures 2 and 3. At the point when SDF level was relatively higher (15%≤DFI<25%), the fertilization, good quality embryos formation, embryos implantation and clinical pregnancy in IVF treatment were altogether diminished contrasted and ICSI treatment (P<0.05) (Figure 4**).**

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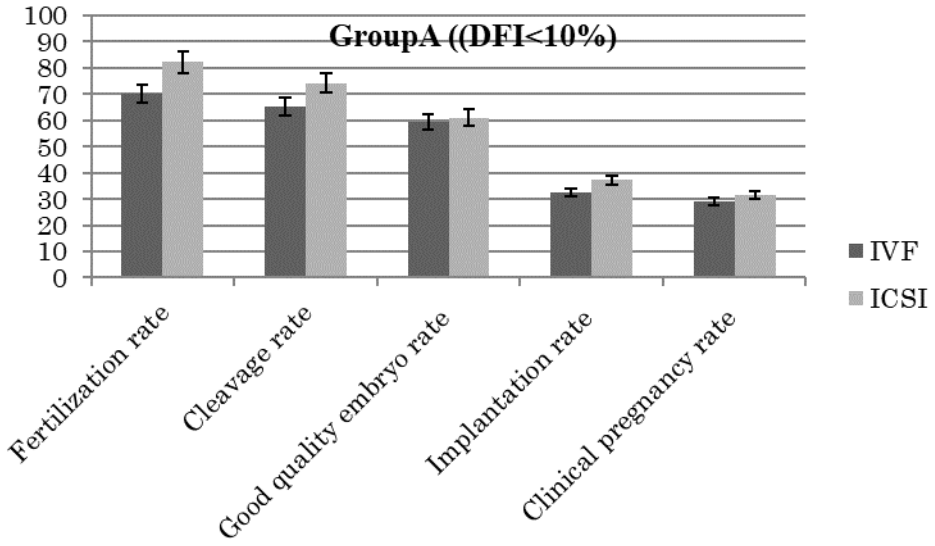

Figure 2: Embryo outcome with IVF and ICSI at DFI Group A (DFI<10%)

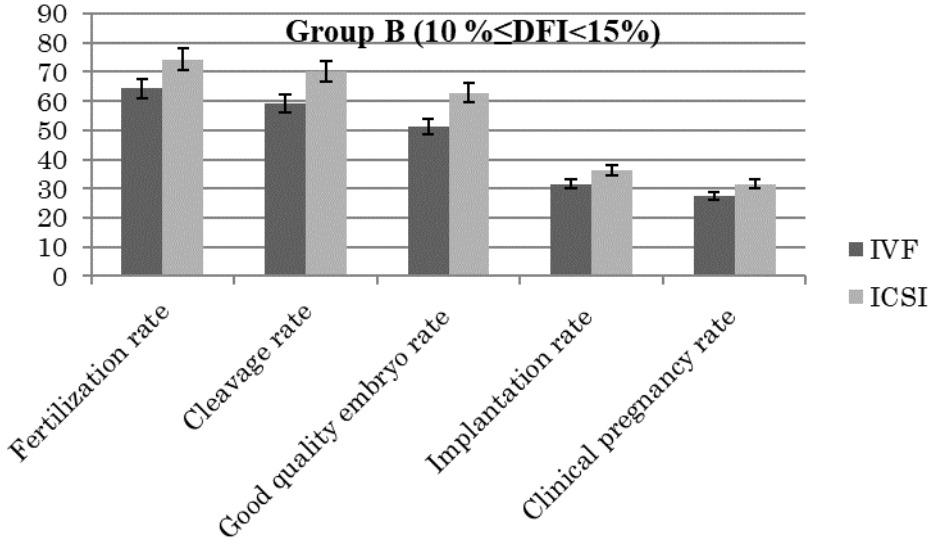

Figure 3: Embryo outcome with IVF and ICSI at DFI Group B (10 %≤DFI<15%)

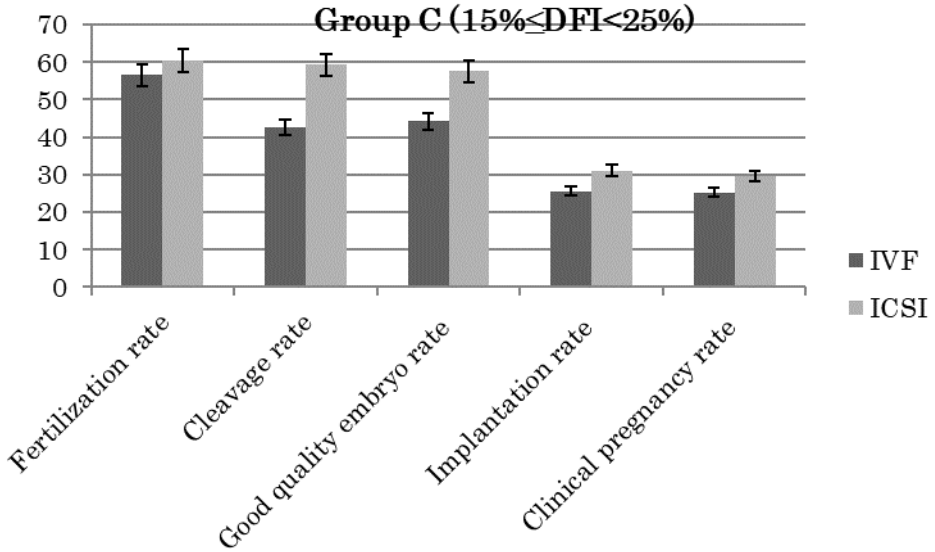

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Figure 4: Embryo outcome with IVF and ICSI at DFI Group C (15%≤DFI<25%)

4. DISCUSSION

SDF is a sort of deformity of the hereditary integrity of sperm and is a substantial marker for the evaluation of male infertility [16]. Various investigations have affirmed that sperm DNA damage can anticipate the treatment results of ART cycles [17]. A few examinations have proposed that the increase index of DFI can decrease the motility and concentration of sperms and change the morphology of sperms. The possible reason is that the abnormal level of SDF prompts the change of the quality of the sperm Na+-K+-ATPase, which causes the denaturation of ATPase lastly results in the decrease of sperm motility, sperm concentration and morphology [18].Along these lines, it tends to be presumed that SDF levels are contrarily correlated with sperm motility. Nonetheless, this study did not agree with this point. Our study found that the DFI estimation of ICSI cycle was higher than that of IVF cycle, and the relative sperm concentration, sperm motility normal morphology were somewhat lower than the IVF cycle, yet the difference was not statistically significant, recommending that sperm DNA damage may not essentially change the general parameters of sperms, which was unique in relation to the consequences of past looks into. It was speculated that the event of various outcomes may be identified with the distinctive patients enlisted in the study [19]. To start with, the sample size of this study was small and concerning couples who chosen ART treatment principally due to the oviduct factors, the connection amongst DFI and sperm parameters probably won't exist. In addition, the study of this analysis depended on the level of DFI; therefore, despite the fact that the anomalous DFI may prompt the difference in general parameters of spermatozoa. Subsequently, the after-effects of this investigation may be one-sided to some degree. Furthermore, further investigations with bigger example size ought to be done to recognize the connection amongst DFI and sperm parameters and determine the criteria of the normal range ofDFI [20]. The DFI estimation of the sperm tests wasmainly distributed between 10 % to 25% in the 290 ARTcyclesofthisstudy, while it was predominantly distributed somewhere in the range of 0% and 10% in ART cycles of studies by Simon et al., which may be principally caused by various SDF detection strategies [21-23].Various SDF detection techniques have different SDF index for the given samplesIn general the detection of the sperm DNA fragmentation TUNEL is considered the most reliable method to judge the DFI [24].At present, there is no clear decision about the impact of sperm DNA damage on the ART cycles. A few investigations have recommended that SDF just influence the result of ICSI treatment [25], in light of the fact that the head part of sperms with DNA fragmentation can be expanded when treated with ICSI.As in the IVF treatment, the zona pellucida of oocyte can naturally distinguish and screen out those sperms with serious DNA lack, in this way, the chosen sperms are generally the ones without damage or with minimal fragmented DNA in this way, it won't influence by SDF [26]. However in this investigation, it was discovered that there were nosignificantdifferences in the rates of fertilization, embryo cleavage, embryo implantation, clinical pregnancy among ICSI and IVF cycles when SDF level was low. But when DFI level surpassed 15%, the rates of rates of fertilization, embryo cleavage, embryo implantation, clinical pregnancy in ICSI cycles were fundamentally higher than those in IVF cycles. In this manner, this investigation recommends that the effect of SDF on the treatment of ART cycles might be among a specific edge [27]. At the point when the SDF level is underneath the threshold, there is no significant difference among ICSI and IVF treatment results, when the SDF level surpasses it, SDF prominently affects the embryonic outcomes to ICSI and IVF, and ICSI outcome is better than IVF. Zini et al [28]. additionally affirmed that, when the sperm DFI value increase 27%, biochemical

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pregnancy rate of ICSI treatment was fundamentally higher than that of IVF treatment, while when the sperm DFI surpassed 30%, clinical pregnancy rate and live birth rate in ICSI treatment were clearly higher than those in IVF treatment [29].In such manner, Zhang et al. [30] contended that ICSI treatment could make sperms omit the common fertilization process and specifically got into the oocyte, and after that fertilized with oocytes typically, despite the fact that these sperms had DNA deformity. After fertilization, oocytes could fix damaged sperm DNA. Hence, the fertilization rate of ICSI treatment would not be influenced. In any case, the embryonic results of IVF treatment would be influenced by the disappointment of failed fertilization on account of serious sperm DNA damage, or influenced by the strange fertilization when the level of sperm DNA damage after treatment far surpassed the fix limit of oocytes [31].Henceforth, when SDF surpassed an abnormal threshold, the endured damage of sperm DNA in ICSI treatment was not as much as that in IVF treatment and its treatment result was superior to that of IVF. Thus, when sperm DNA fragmentation was not serious and DFI was moderately higher, ICSI could accomplish a superior clinical result than IVF. Our study also revealed that, irrespective of different levels of sperm DNA damage, there was nosignificantdifferenceingoodqualityembryo rate between the treatment of ICSI and IVF, demonstrating that sperm DNA fragmentation did not affect the good quality embryo rate of ART. However, most investigations have demonstrated that sperm DNA fragmentation are in negative relationship with goodqualityembryorateofART[32-33], which isdifferent in theresultofthisstudy.(table:4 and fig:5)

Table 4: Assessment of IVF and ICSI on Implantation and clinical pregnancy rate with 3 groups DFI.

Group IVF-ICSI-SDF	Implantation rate	Clinical pregnancy rate
IVF (DFI <10%)	32.5	28.9
ICSI (DFI <10 %)	37.2	31.6
IVF (10%<DFI<15%)	31.5	27.5
ICSI (10%<DFI<15%)	36.4	31.5
IVF (15%< DFI<25%)	25.6	25.2
ICSI (15%< DFI<25%)	31	29.6

Note: Data presented as percentage (%)

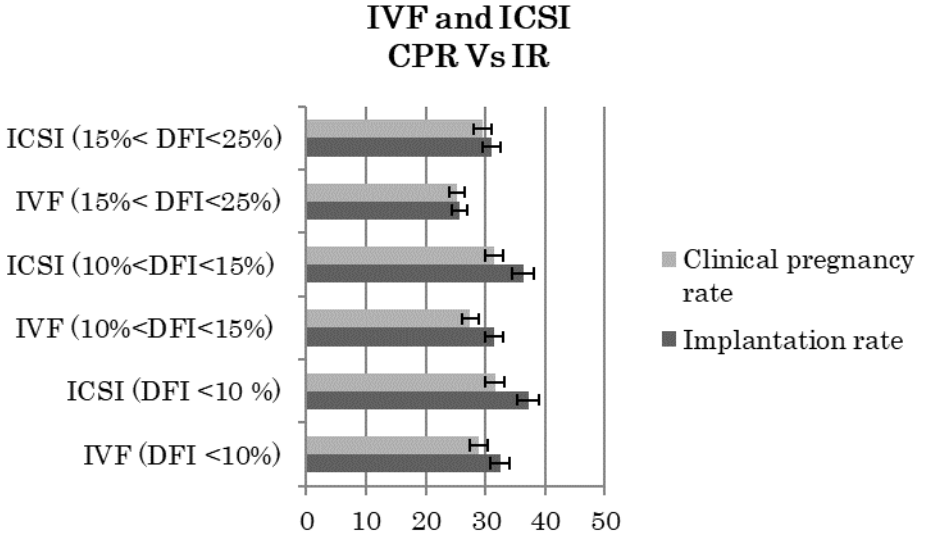

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Figure 5: IVF and ICSI on Implantation and clinical pregnancy rate with 3 groups DFI

5. CONCLUSION

The impact of SDF on the clinical result of ICSI and IVF is identified with threshold. When sperm DNA damage is high there is a decrease in the pregnancy and implantation rate in the ART cycles however the level of treatment is better in the ICSI cycles than IVF with high DFI index. Therefore, ICSI treatmentshouldbechosenwhenthelevelofSDFis relatively high in ARTtreatment.

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PHARMACOGENOMIC APPROACH AS PERSONALIZED MEDICINE FOR TYPE 2 DIABETES MELLITUS

Gouthami K[1], Veeraraghavan V[1], Vani Puttaraju[2], Prashantha CN[3], Ramachandra R[3]
[1]Department of Biochemistry, School of Applied Sciences, REVA University, Bangalore-560064
[2]Department of Biotechnology, Dayananda Sagar College of Engineering, Bengaluru- 560078
[3]Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560064

ABSTRACT: Hyperglycemia is a metabolic disorder that is commonly observed in 90% of population in all over the world. Many genetic variants are associated with disease and cause several risk factors. In the current study is targeting on Indian population having hyperglycemia and the role of pharmacogenomic properties of drugs within this population. Based on the genetic study within Indian population having very less number of genetic variants in hyperglycemia but molecular mechanism of disease progression and drug response remain unknown. Using pharmacogenomic approaches there are several anti-diabetic drugs need to study and screen based on type of genetic variant to predict personalized medicine. There are many anti-diabetic drugs is not equally effective on all patients. Here, we performed Genome-wide association study (GWAS) to predict the pharmacogenomic properties of drugs that are used for treatment for personalized medicine. There are several insilico drug discovery methods is used to understand pharmacophore and pharmacokinetic approaches also to predict drug docking interactions. The results of anti-diabetic drug such as metformin, glyburide, Acarbose, pioglitazone and voglibose compounds is more effective with HNF1A, HNF1B, TCF7L2 and APM1. By using this approach, further the researchers and clinicians need to test to clinical approaches that has significant within individual population to predict as a personalized medicine.

Keywords: Hyperglycemia, Pharmacogenomics, Pharmacogenetics, drug design, diabetes mellitus

***Corresponding Author:** Gouthami K.
Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560064, Email: gouthamik@reva.edu.in

1. INTRODUCTION

Hyperglycemia or type 2 diabetes mellitus is a world 1st largest disease having 90% of population is affecting the disease [1]. A current estimate indicates that 442 million people in global prevalence of diabetes, it representing 8.5% of the population, and close to 500 million people worldwide [2]. Well established risk factors of diabetes include impaired glucose tolerance and impaired fating glucose is independent, hypertension, obesity and heart problems [3]. However, a significant number genetic mutation that influences hyperglycemia, the role of genetics has been evidenced through studies on family history and life style of the people. There are 36 genes have been reported in worldwide population till 2011 [4]. But the rate of hyperglycemia is more in Indian population. MODY has classified the clinical criteria of Indian population with worldwide population study shows only 23 gene mutations were observed in Indian population [5].

The systematic study of Indian populations shows mutations on HNF1A, HNF1B, PPARG, TCF7L2 and APM1 gene mutations are most common in South Indian population [6-10]. The HNF1A has several variants subjected to the normal glucose tolerance to the novel mutations

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538G>C at promoter region and six novel coding region mutations namely Arg114Cys, Val134Val, Arg171Gly, Glu235Gln, Gly245Arg and Arg263His. Another novel polymorphism such as 373C<T at promoter region and Val103Met at coding regions in HNF1A [11]. The HNF1B has three variants -67C>T, Arg165His and IVS2+2insT and two novel variants IVS3nt – 4C>G, Asn321Asp. The heterozygous whole gene deletion of exons 1-9 (Met1_Trp557del were observed in two patiens. The novel variation Asn321Asp was found in heterozygous of whole gene deletions [12]. The PPARG gene has several genetic variants affects expression levels of targets genes and these gene variants such as Pro12Ala, C1431T, Pro495Leu, Val318Met, Phe388Leu and Arg425Cys known variants, P12A is the novel gene variant present in Indian population [13]. TCF7L2 genes are association of a variant of the gene, rs7903146 and rs12255372 (G/T) polymorphism is observed in south Indian populations. There are several gene mutations such as Asn50Tyr, Pro202his, Lys346Asn, Ala379Tyr, Pro483Tyr, Pro495Arg and Ser509Pro [14]. APM1 gene has positive association of polymorphism between SNP45 T > G in Indian population and SNP276G > T (Arg112Cys) in Japanese population. The other genetic mutations −11391G > A, −1377C > G [promoter] and 45T > G [exon 2] and 276G > T [intron 2] is increases the risk of coronary heart disease [15].

Commonly used antidiabetic drugs such as Insulin is a major component in optimizing metabolic functions [16]. There are other types of oral selected drug compounds such as biguanides, Thiazolidinediones, secretagogues, alpha-glucosidase inhibitors and peptide analogs is provided to the patients that depends on the type of risk factor and symptoms [17]. There are several compositions in each oral drug such as biguanides decreases blood glucose level and they decrease intestinal glucose absorption, the common compounds of this group such as metformin, Phenformin and Buformin is used to treat diabetes [18]. There are more than 15 other compounds also involved in this group. The Thiazolidinediones inhibitors binds to PPARG gene of nuclear regulatory protein and the common compound in this group is rosiglitazone and pioglitazone [19]. The secretagogues drugs increase insulin secretion in pancrease, this group of compounds such as sulfonylureas property compounds include tolbutamide, acetohexamide, tolazamide, chloropropamide, glipizide, glibenclamide, and 22 compounds is belongs to this group [20]. Alpha-glucosidase inhibitors reduce glucose absorption in small intestine these compounds such as Acarbose, miglitol and voglibose these compounds decreases the production of enzymes needed to digest carbohydrates [21]. In the present research we are selecting few compounds such as metformin, glyburide, Acarbose, pioglitazone and voglibose to understand pharmacogenomic property with types 2 diabetic proteins [22]. Insilico drug discovery methods are used to study biological active molecules against target protein structures to screen biologically active molecules against the disease condition.

2. MATERIALS AND METHODS

In the present study is to identify the pharmacogenetic and pharmacogenomic properties of types 2 diabetes and their medications. There is no clear information on how genetic transformation will affect T2D[23]. Only proof is that single nucleotide polymorphism (SNP) is associated with increased risk of T2D. To identify the gene position and mutational loci of different risk factors associated with T2D [24]. The Genome Wide association study (GWAS) is used to compare whole genome sequence associated with diabetic patient group and non-diabetic control groups to predict genetic variants. There are 65 different genetic variants were predicted in worldwide population, but within Indian population only 21 genes is associated in T2D. Here we include HNF1A, HNF1B, PPARG, KCNJ11, ABCC8, TCF7L2, APM1, SLC30A8, WFS1, HNF4A, CDKN2A, CDKN2B and HHEX gene is most commonly

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observed in both Indian and other populations. Few genes such as IPF1, ENPP1, PGC1A, IRS2, IAPP, IRS1, GLUT4 and CAPN10 genes only observed in T2D. Here, we have selected six major target genes and their genetic variants in Indian and world-wide population HNF1A, HNF1B, PPARG, TCF7L2, and APM1 genes are used to screen pharmacogenetic and pharmacogenomic properties [25].

2.1 Protein structure prediction

The T2D mutated target protein sequences were used to predict the 3dimentional protein structure using Swiss model and PSI-BLAST and the resultant templates were used to build the 3D structure. After 3D protein structure to mutate the amino acid positions where the gene and protein have genetic variants and the resultant protein structure is used for structure validation by SAVS and active site prediction by CastP calculation server. CastP used to calculate topological surface area and surface volume to understand electrostatic interaction energy and also with hydrophobic and hydrophilic groups arranges in amino acid pockets.

2.2. Ligand molecule selection

The commonly available antidiabetic drugs such as biguanides, Thiazolidinediones, secretagogues, alpha-glucosidase inhibitors and peptide analogs were retrieved from Pubchem compound database. The formulated ligand molecules were modified based on physical and chemical properties, using pharmacokinetic properties include structural screening, fragment-analysis and SAR facilitated to probe the parent library. The fragments were identified on the basis of “Lipinski's Rule of Five” that represented a suitable strive for proficient lead compounds. The training sets of lead molecules were generated through conformational search module and further implementation has been done by Hyperchem Professional 7.0. For each compound, the systematic conformational search was attained by energy minimization by starting with 1000 to 2000 initial geometries at random torsion angle values. These were exclusively described about exocyclic single bonds and chemical bonds within nonaromatic cycles. These ligand molecules can be used as potential drug molecules.

2.3. Molecular Docking and virtual screening

After protein structure and ligand property validations are carried out, molecular docking study is done using one of the best docking tools AutoDock 4.2. To the ligand segments polar hydrogen was inserted and assignment of Gasteiger type was also done. To the carbon atoms nonpolar hydrogen bonds were combined. Then torsion values and also internal degrees of freedom were fixed. For this docking experiment a collection of algorithm such as Lamarckian genetic algorithm was used. Other parameters such as mutation rate, cross over rate, population size were fixed to 0.02, 0.8 and 150 respectively. The simulations were performed until the required energy values are obtained. All the simulations were carried for 10 times and from this 10 protein ligand interaction confirmations were formed. Out of these 10 resulted confirmations, the confirmation having lowest energy was considered. Finally based on the binding energy between the proteins and ligand molecules, the docking studies between the normal and mutated protein structures were compared to analyze the effect of the drug. Using virtual screening methods to screen the compounds based on interaction energy between protein and ligand that helps to understand how best the drug activity against the protein and also to understand the side effect will cause with very strong interaction energy.

3. RESULTS AND DISCUSSION

First we have analyzed the normal and mutant protein interactions of most significant non synonymous SNPs of HNF1A, HNF1B, PPARG, TCF7L2, and APM1 genes with antidiabetic drug classes such as Sulfonylureas (Glyburide), biguanides (Metformin), Thiazolidinediones (Pioglitazone), Alpha-glucosidase inhibitor (Acarbose) and Glycoge-like peptide 1(GLP-1) (Voglibose) compounds were used to performing pharmacogenomic studies and molecular

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docking techniques. In the second step we have predicted the pharmacophore properties followed the "Lipinski rule 5" based on energy minimization with improved chemical and physical properties. The target proteins were analyzed the hydrogen bond interaction energy values. Finally the difference in the energy values between the commonly available drug molecules were calculated and select best drugs for T2D treatment. We have screened the drug compounds based on normal and mutant protein interactions. So that these drug molecules can be prescribed to those without or with bearing these gene variants.

The target sequences of HNF1A, HNF1B, PPARG, TCF7L2, and APM1 proteins with structurally predicted templates include, 1ic8, 2h8r, 3dzu, 2lef, and 4dou were used to predict the three dimensional structures of normal and mutant proteins and these structures were used for homology modeling and the resultant protein structures is used for structure validation (table: 1). The active site amino acids of both normal and mutated protein structures is predicted by CastP to calculate surface area and surface volume to understand the drug binding sites. Here, we use mutated sites as ligand binding sites that can predict molecular docking. The resultant protein structures were used to find the active site amino acids and to calculate the electrostatic and van-der-Waals interactions between residues of complex proteins. The active site amino acids were identified based on RMSD values.

Table: 1. Target protein structures and template to predict 3D complex protein structure using Swiss model.

Protein	Template	Quality score	Verify3D	Z-score	Q-mean	Ramachandran Plot
HNF1A	1IC8	73.656	73.71%	0.14	-5.86	83.1%
HNF1B	2H8R	54.500	64.09%	0.20	-2.80	91.5%
PPARG	3DZU	74.652	74.59%	0.68	-4.25	78.5%
TCF7L2	2LEF	89.062	50.00%	0.08	-0.02	85.1%
APM1	4DOU	73.282	96.43%	0.60	-1.95	81.3%

3.1. Pharmacological analysis of anti-diabetic drug compounds

The pharmacophore properties of antidiabetic drug compounds were retrieved from Pubchem compound database and Lipinski rule is predicted by molinspiration (table: 2). The QSAR properties of antidiabetic compounds are predicted based on biological properties using Hyperchem 7.5 Professional. We have calculated the semi-empirical properties with geometrical optimization predicts QSAR properties such as Surface Area Approx (SAA), Surface Area Grid (SAG), Volume (VOL), Hydration Energy (HE), Log P (Log P), Refractivity (RF), Polarizability (POL),Mass (Mass) is used as dependent variable in QSAR study (Table: 3). The resultant ligand molecules were used for molecular docking. The molecular docking of active site receptor proteins were performed using AutoDock program and has been shown to successfully reproduce experimentally observed binding modes in terms of lowest docking energy.

Table: 2. Pharmacophore properties of selected ligand molecules

Ligands	logP	TPSA	nAtoms	MW	HBA	HBD	Nrotb	volume	Violations
Metformin	-1.13	88.99	9	129.17	5	5	3	126.83	0
Glyburide	4.77	113.60	33	494.01	8	3	8	424.74	0
Acarbose	-5.51	321.16	44	645.61	19	14	9	544.93	3
Pioglitazone	3.07	68.30	25	356.45	5	1	7	318.53	0
Voglibose	-3.98	153.62	18	267.28	8	8	5	238.17	1

Table: 3. QSAR Properties of Synthetic compounds predicted using Hyperchem 7.0

Compounds	**Metformin**	**Glyburide**	**Acarbose**	**Pioglitazone**	**Voglibose**

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SAA ($Å^2$)	183.48	874.77	765.43	706.85	471.68
SAG ($Å^2$)	289.63	701.20	805.28	587.88	399.25
Vol ($Å^3$)	410.04	1180.67	1368.74	949.41	627.35
HE (kcal/mol)	-18.71	-6.74	-28.64	-5.12	-11.15
LogP	1.75	-0.52	21.34	1.67	10.68
RF ($Å^3$)	18.00	106.22	53.47	84.44	32.07
POL ($Å^3$)	7.57	37.10	43.78	30.41	17.97
Mass (amu)	118.08	465.78	602.27	336.28	246.11

3.2 Molecular docking

In order to predict the docking of most routine protein-ligand interactions is carrying out of HNF1A, HNF1B, PPARG, TCF7L2 and APM1 proteins as well as to potential ligands of already in used compounds are compared in this work. The molecular docking is performed by AutoDock 4.2. The estimated ligand binding energy, number of hydrogen bonds formation, estimated inhibitory constant (Ki) and amino acid binding sites that helps to understand routine returned top ten ranked docked poses for each ligand and the number of docking poses is summarized in table: 4a

A) .The site-directed mutagenesis of HNF1A within Indian population is used to predict the ligand binding sites that can help as anti-diabetic properties. The comparison between normal HNF1A control proteins with HNF1A mutated protein is posed with experimentally identified ligand binding pocket residues. In the both the proteins has 35 pocket sites of which 27 active site amino acids (table: 4a), the binding energy of -3.6, -3.79, -2.88, -2.47 and -3.24 kcal/mol of estimated energy is strong covalent interaction with 4-8 hydrogen bonds within active site amino acids His126, Arg131, Glu132, Lys169, Gln176, Gly191, Asn202, Arg203, Trp206, Arg244, Gly253, Leu254, Ser256, Asn257, Arg263 and Arg265. The mutated protein is strong binding with ligands by forming -6.07, -5.91, -2.74, -2.25 and -3.47 kcal/mol of estimated binding energy by forming 4-13 hydrogen bonds within active site amino acids Glu172, Gln176, Leu254, Arg168, Lys169, Lys197, Glu110, Tyr166, Val173, Tyr122, Arg244, Pro195, Asn202, Gly253, Ser256 and Asn257 and these compounds is best therapeutic for anti-diabetic drug molecule. The overall results shows the ligand structures within normal protein is showing very good effective and no any objections but in mutated protein voglibose compound showing 13 hydrogen bonds with strong binding energy . The overall results predicts that the voglibose a alpha-glucose inhibitor is showing more effective and in some conditions it may be affect some side effects .

Table: 4a. Molecular docking of normal and mutated HNF1A protein structures with anti-diabetic drugs

Ligands	Normal Protein				Mutated Protein			
	No. of H Bonds	Binding Energy	Ki (□M)	Amino acids	No. of H Bonds	Binding Energy	Ki (□M)	Amino Acids
Metformin	4	-3.6	2.82	Glu132, Gln176, Gly253, Leu254	4	-6.07	1.65	Gly191, Gly253, Leu254, Gly255
Glyburide	4	-3.79	9.45	Arg203, Ser256, Arg263	4	-5.91	119.2	Thr136, Arg168, Lys169, Lys197
Acarbose	5	-2.88	26.48	Arg131, Trp206, Arg265	4	-2.74	34.12	Glu110, Tyr166, Val173
Pioglitazone	4	-2.47	1.63	His126, Lys169, Gly191, Asn202	2	-2.25	109.7	Tyr122, Arg244
Voglibose	8	-3.24	14.22	Glu132, Asn202, Ser256, Asn257,	13	-3.47	1.78	Glu172, Gln176, Pro195, Asn202,

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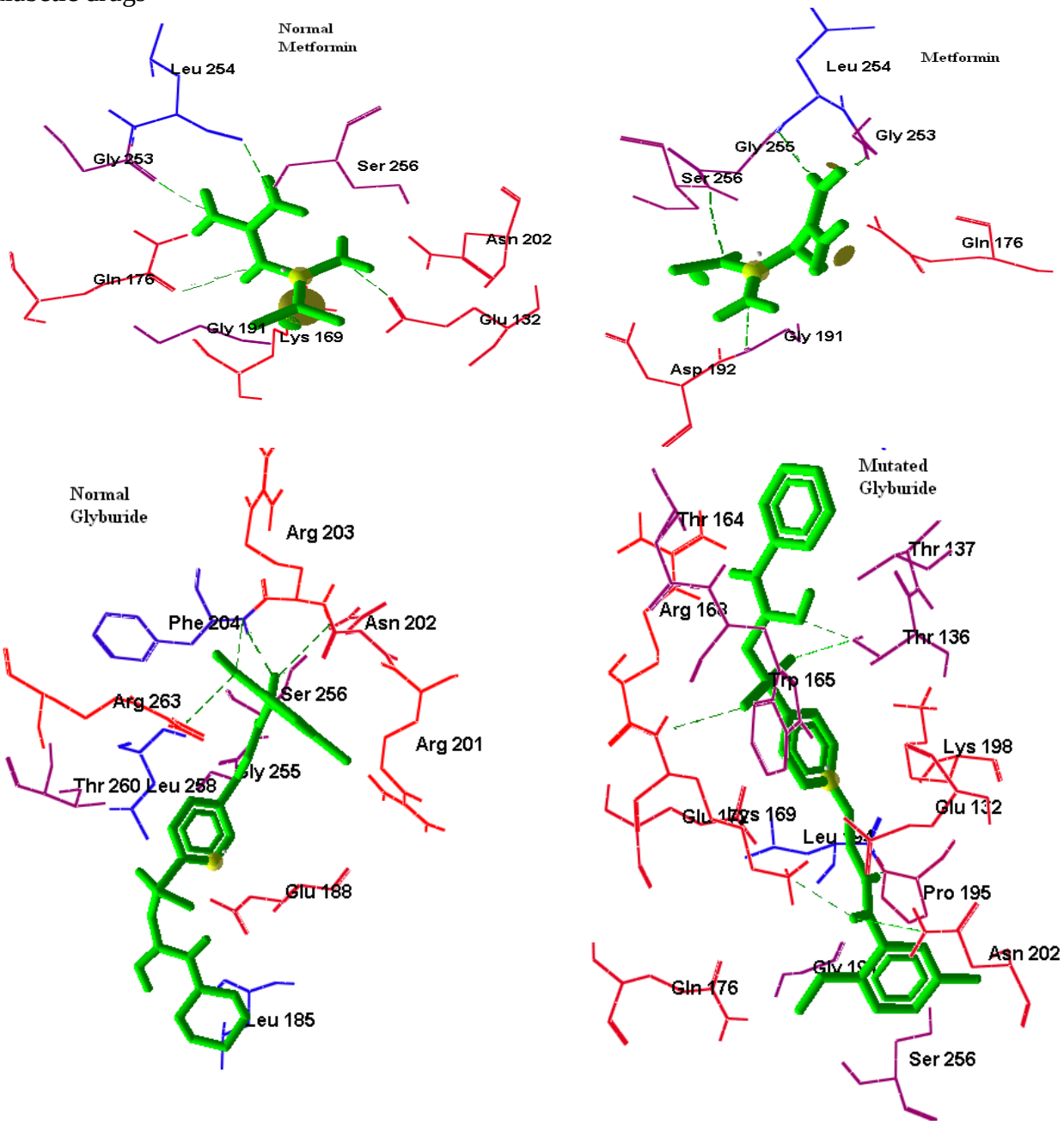
Leu254, Arg244 Gly253, Arg244, Ser256, Asn257,

Figure: 4a Molecular docking of normal and mutated HNF1A protein structures with anti-diabetic drugs



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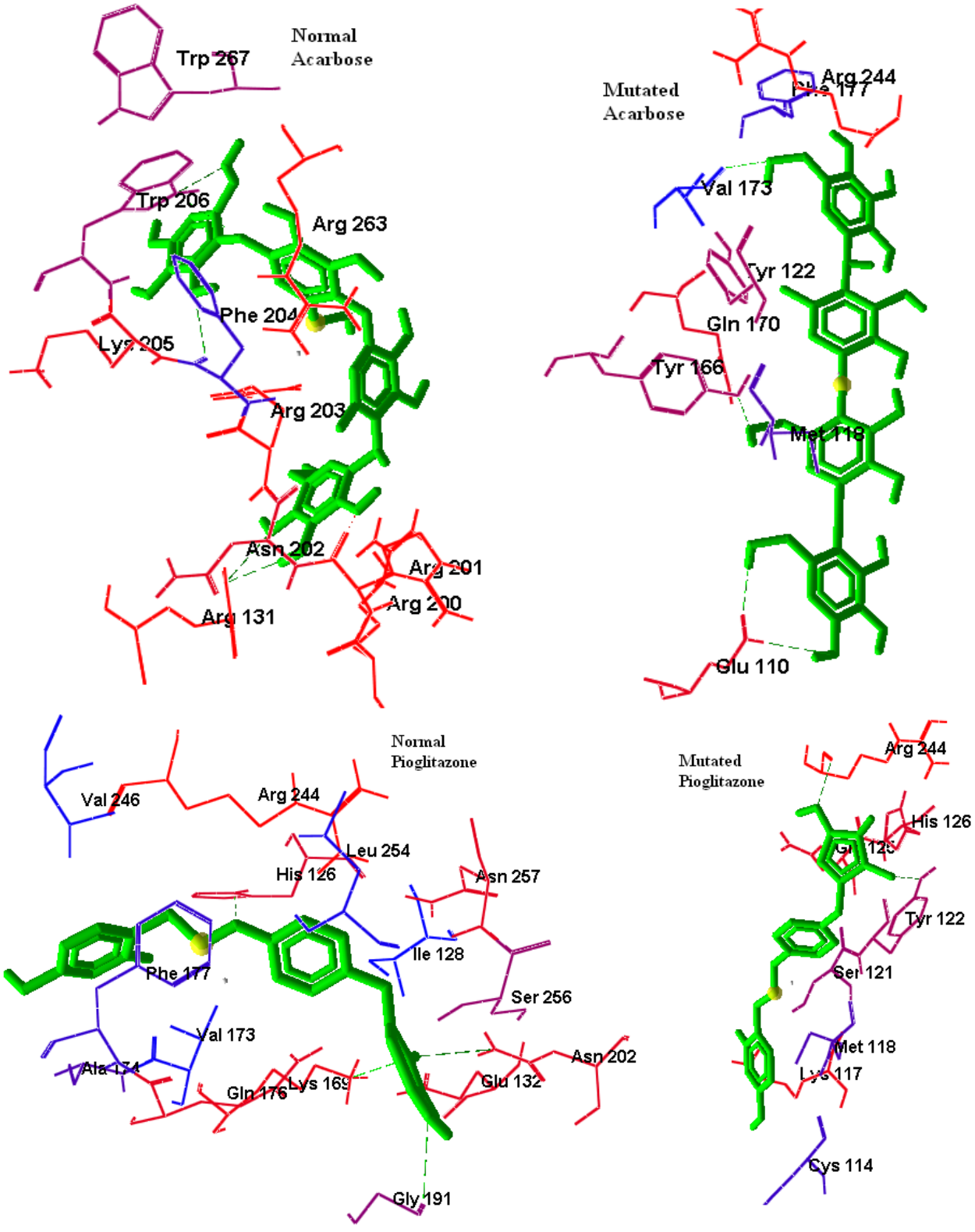

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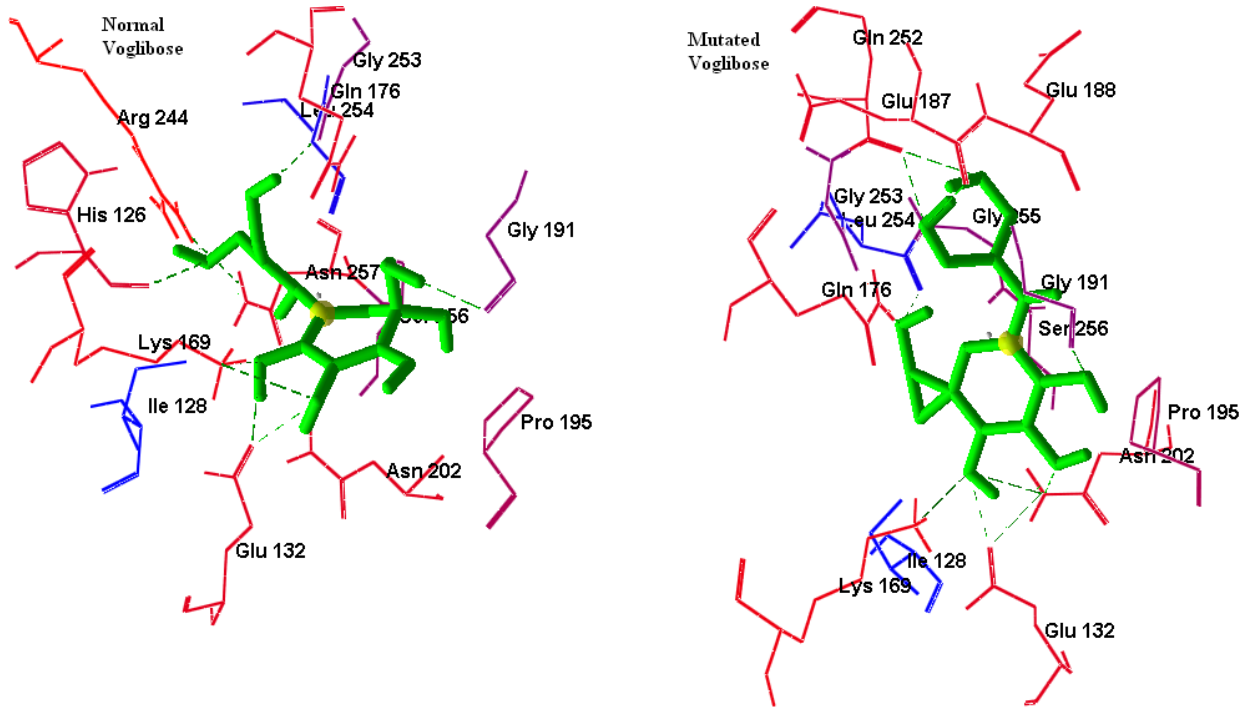

B) . The HNF1B protein having mutational site at Arg165His and Asn321Asp region is predicted in Indian Population, further we have predicted the complex structure to predict the active site amino acids shows 23 pockets of 27 active site amino acids. The normal protein structure of HNF1B protein is strong binding energy of -5.47, -4.85, -2.51, -2.48 and -3.11 kcal/mol by forming 4-7 hydrogen bonds within active site amino acids Gln182, Gln188, Ser189, Asn192, Met193, Asn192, Met193, Gln201, Glu208, Phe209, Pro226, Asn228, Lys230, Arg233, Gly285, Leu286, Ser288 and Asn289. The mutated protein is docked with ligand compounds by forming 2-7 hydrogen bonds within active site amino acids Val140, Gln176, Arg181, Gln182, Thr186, Ser189, Ser190, Met193, Arg233, Gly255 and Ser256 of estimated binding energy of -6.231, -6.601, -3.18, -6.166 and -1.81 kcal/mol. The overall results shows all the compounds is best anti-diabetic drug molecules .

Table: 4b. Molecular docking of normal and mutated HNF1B protein structures with anti-diabetic drugs

Ligand	Normal Protein				Mutated Protein			
	No. of H Bonds	Binding Energy	Ki (□M)	Amino acids	No. of H Bonds	Binding Energy	Ki	Amino Acids
Metformin	4	-5.47	12.48	Asn228, Pro226, Phe209, Glu208	3	-6.231	48.1	Ser189, Ser190, Met193, Gln176, Gly255, Ser256
Glyburide	5	-4.85	28.8	Gln182, Gln188, Gly285, Ser288	4	-6.601	68.7	Ser189, Ar233
Acarbose	7	-2.51	20.59	Gln188, Ser189, Asn192, Met193, Gln201, Arg233	4	-3.18	105.78	Arg181, Gln182, Thr186
Pioglitazone	4	-2.48	15.66	Asn192, Lys230, Ser288	2	-6.166	107.38	Glu178, Gln182
Voglibose	7	-3.11	8.8	Gln182, Lys230, Gly285, Leu286, Asn289	7	-1.81	70.97	Lys175, Gln182, Lys230, Leu286, Asn289

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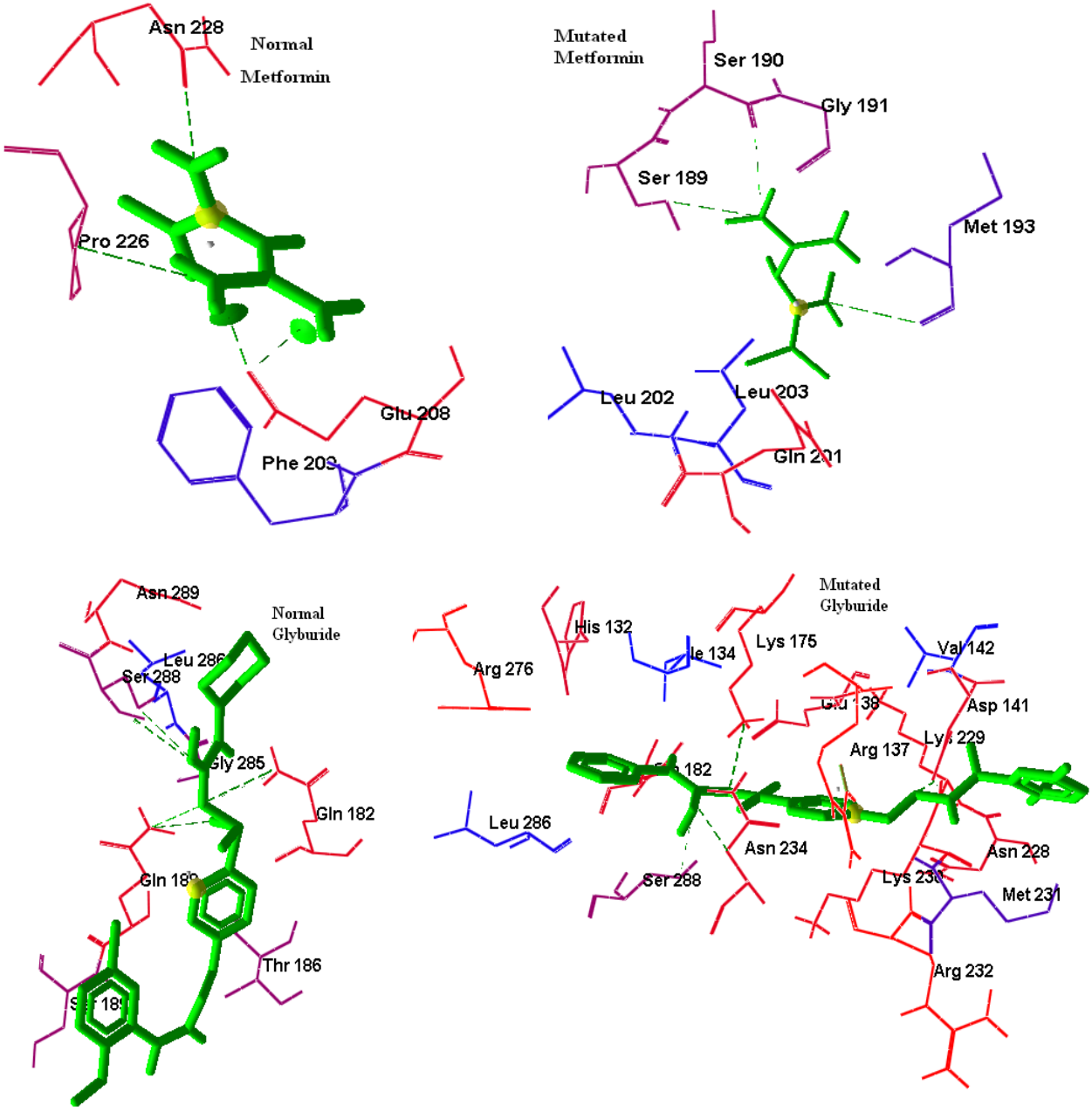
Figure: 4b Molecular docking of normal and mutated HNF1B protein structureswith anti-diabetic drugs.



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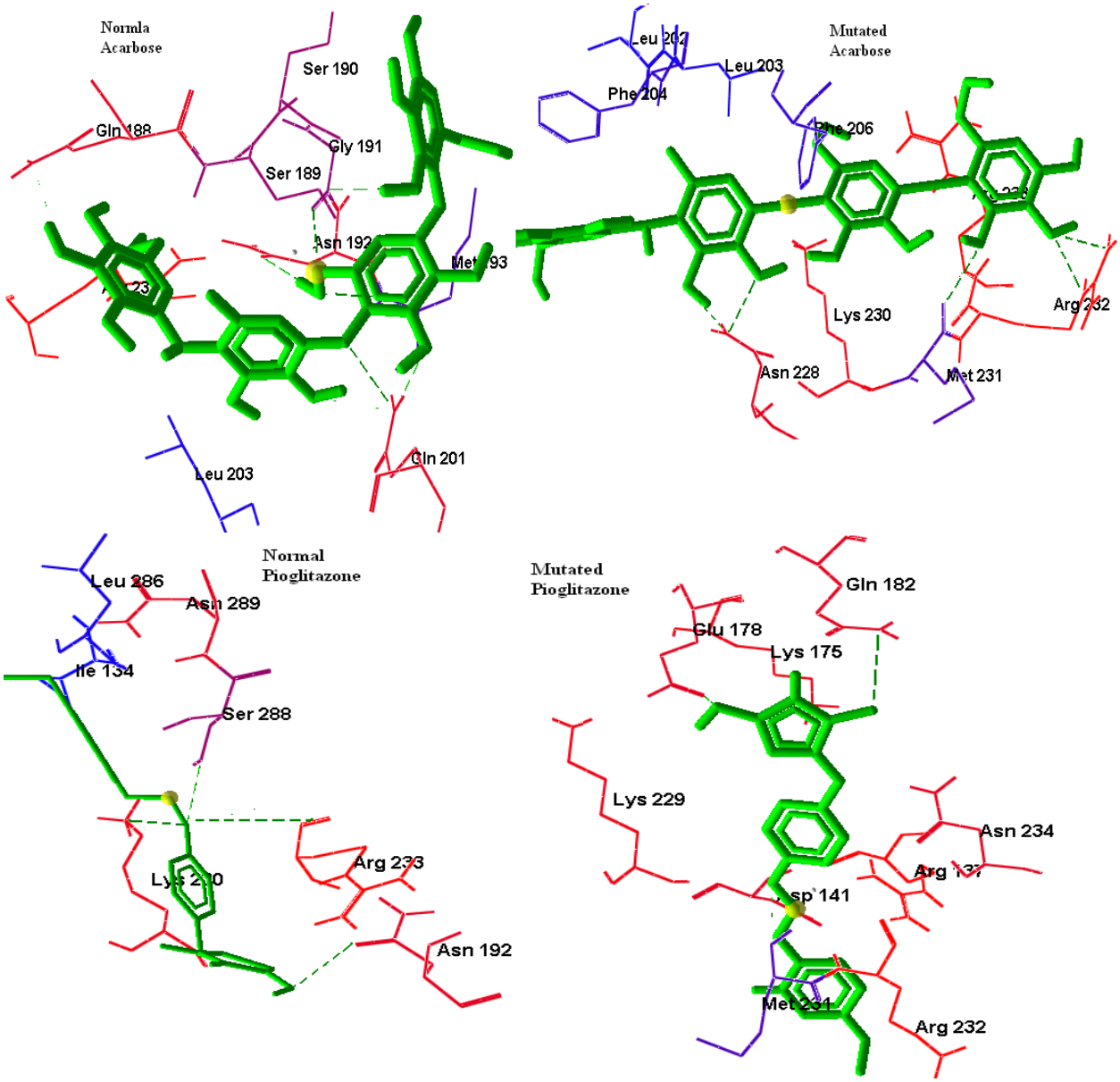

c). The PPARG protein has several gene mutations such as Pro12Ala, Cys1431Tyr, Pro495Leu, Val318Met, Phe388Leu and Arg425Cys amino acids were observed in Indian population. The normal protein and mutated protein structures are done molecular docked within active site amino acids. We have predicted the active site amino acids based on pocket regions, there are 46 pockets of which only 10 amino acids shows active site points within target region is used for drug binding. We have docked the ligand with normal protein of binding energy of -3.43, -1.22, -2.92, -4.61 and -5.62 kcal/mol by forming 1, 3, 5, 6 hydrogen bonds respectively within active site region such as Pro255, Leu256, Lys291, Cys313, Arg316, Glu319, Glu323, Ser370 and Glu371 amino acids. The mutated protein is strong interaction with ligand molecules of energy -7.61, -2.6, -3.82, -4.74 and -6.04 kcal/mol respectively is strong interaction within active site amino acids Phe254, Pro255, Leu256, Asp288, Thr296, Gln299, Arg308, Cys313, Arg316, Ser317, Glu319, Ile369, Ser370, Glu371 and Lys395 by forming 3-6 hydrogen bonds with strong interactions.

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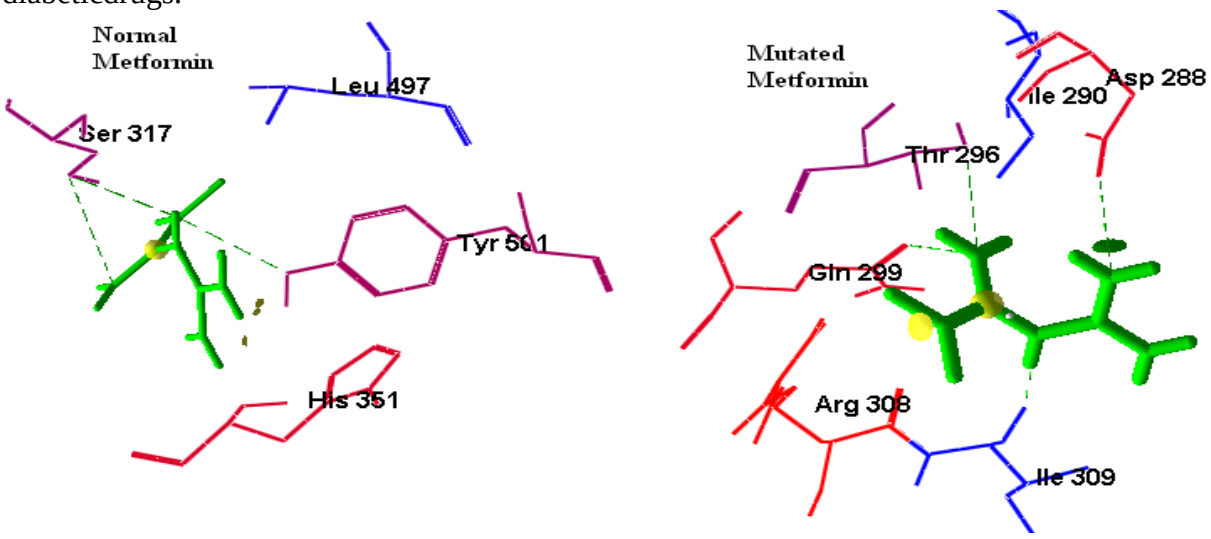
Table: 4c. Molecular docking of normal and mutated PPARG protein structures with anti-diabetic drugs

Ligand	Normal Protein				Mutated Protein			
	No. of H Bonds	Binding Energy	Ki (□M)	Amino acids	No. of H Bonds	Binding Energy	Ki	Amino Acids
Metformin	3	-3.43	7.08	Ser317, Tyr501	6	-7.61	4.26	Asp288, Thr296, Gln299, Arg308,
Glyburide	1	-1.22	25.38	Glu371	4	-2.6	24.53	Leu256, Lys395, Cys313, Ser317
Acarbose	6	-2.92	11.55	Lys291, Arg316, Leu256, Pro255, Glu371	5	-3.82	22.52	Phe254, Pro255, Leu256, Lys395
Pioglitazone	3	-4.61	5.64	Arg316, Glu371, Glu323	4	-4.74	4.32	Ser317, Arg316, Ile369, Ser370, Glu371
Voglibose	5	-5.62	7.02	Leu256, Arg316, Glu323, Glu319	3	-6.04	5.09	Glu319, Arg316

Figure: 4c Molecular docking of normal and mutated PPARG protein structures with anti-diabeticdrugs.



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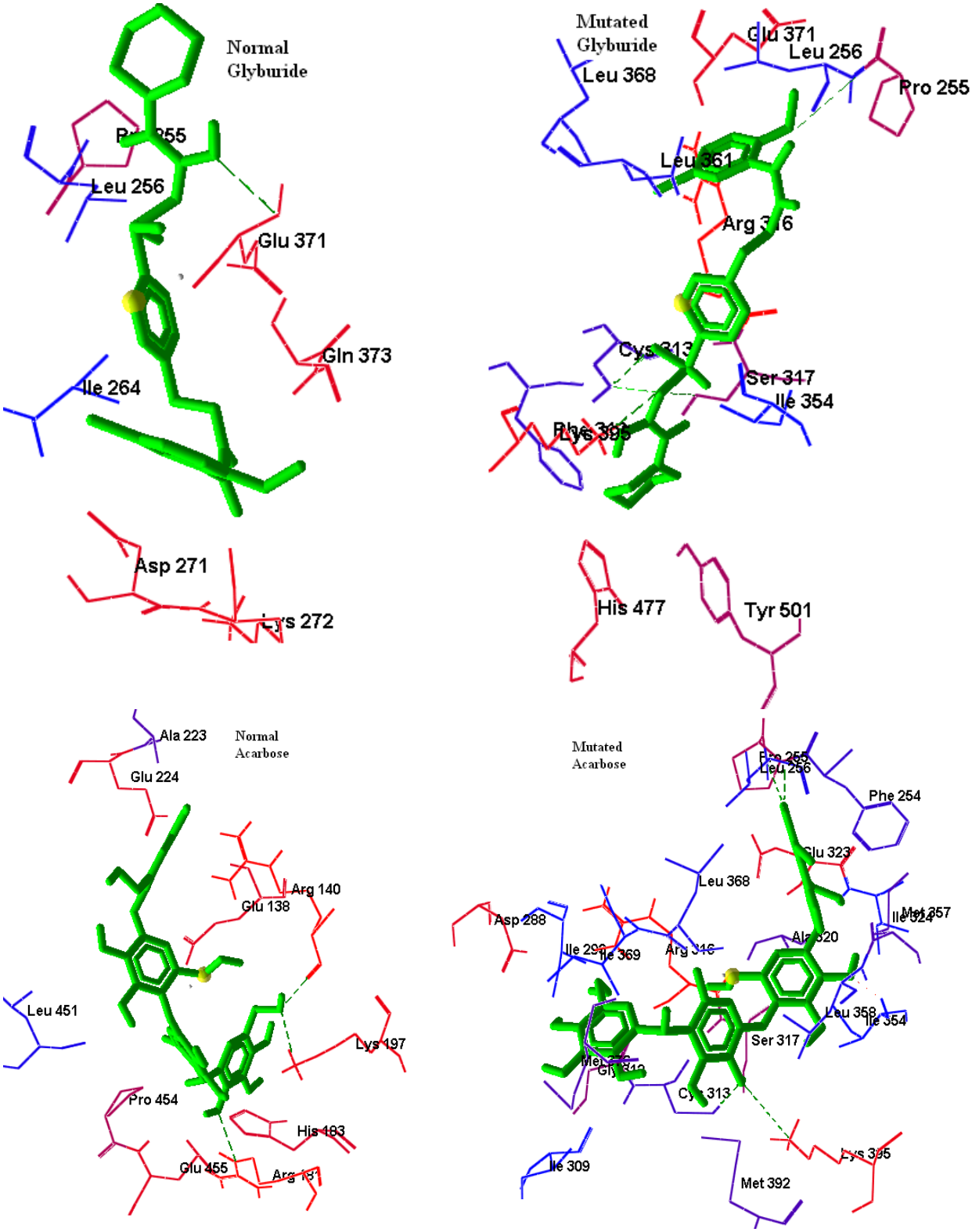

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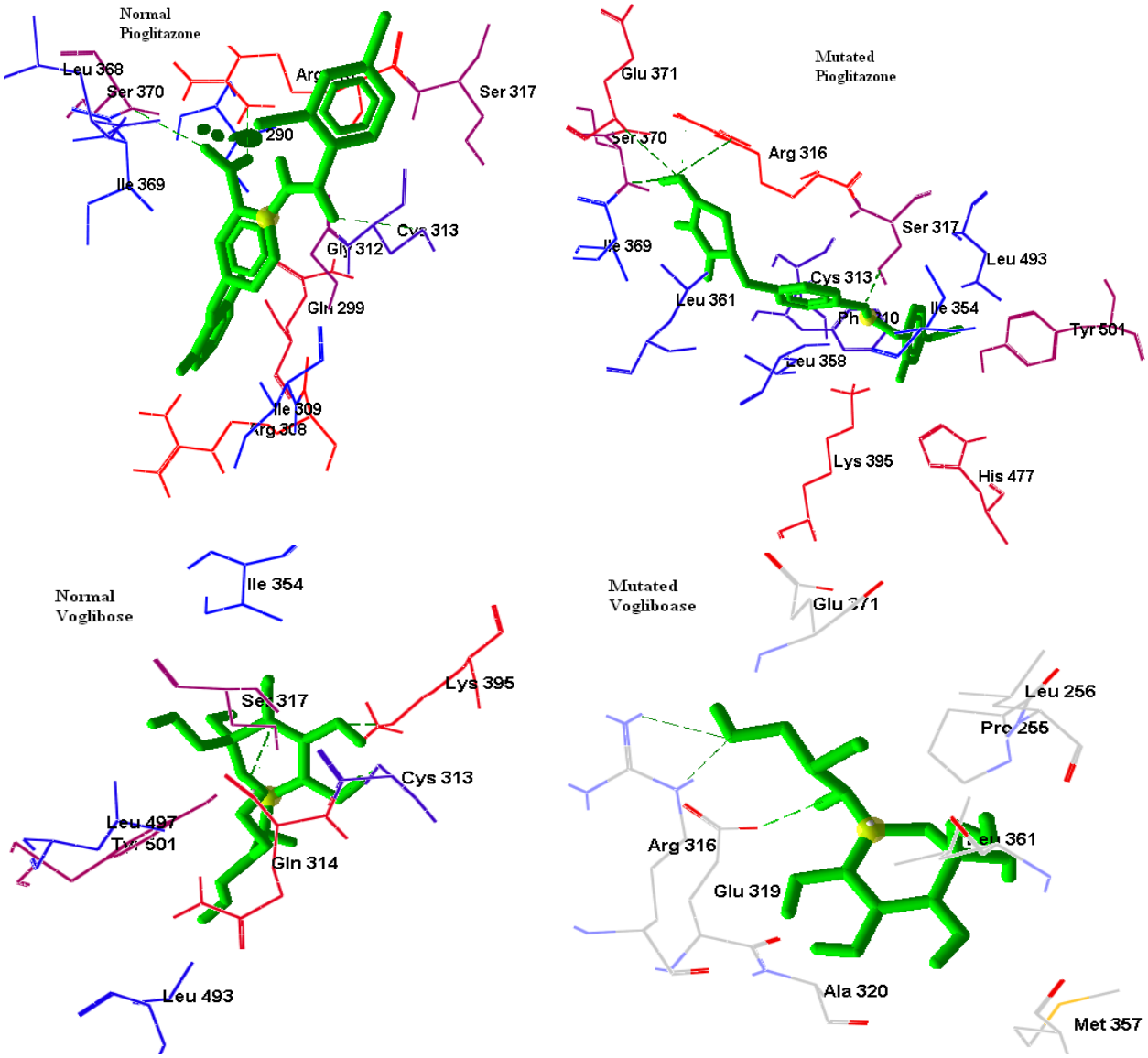

d). The TCF7L2 protein has mutational sites on Ala379Tyr and Pro483Tyr amino acids within Indian population, the active sites is predicted based on number of pockets and amino acids. We have predicted 5 pockets with 8 active site amino acids. Further have done molecular docking with normal protein by forming strong binding energy of -4.02, -3.23, -4.15, -2.55 and -4.68 kcal/mol with in active site amino acids of Pro353, Leu354, Glu406, Arg407, Gln408, His410, Gln412, Trp417, Ala419, Arg420 and Asp421. The interaction of ligand with protein is forming strong covalent bonds of 2-4 hydrogen bonds and these compounds are best molecules on TCF7L2 gene mutations. The mutated protein is strong interaction within mutational sites and within active site amino acids Pro353, Leu354, Glu371, Tyr373, Tyr379, Glu406, His410, Trp417, Arg420 and Asp421 with interaction binding energy of -4.33, -1.04, -2.76, -2.37 and -2.33 kcal/mol respectively shows 2-4 hydrogen bonds to their amino acids.

Table: 4d. Molecular docking of normal and mutated TCF7L2 protein structures with anti-diabetic drugs

Ligand	Normal Protein				Mutated Protein			
	No. of H Bonds	Binding Energy	Ki (□M)	Amino acids	No. of H Bonds	Binding Energy	Ki	Amino Acids
Metformin	3	-4.02	2.08	Arg407, Trp417, Ala419	3	-4.33	2.21	Pro353, His410, Trp417
Glyburide	2	-3.23	25.11	Pro353, Leu354	2	-1.04	17.68	Glu371, Tyr379

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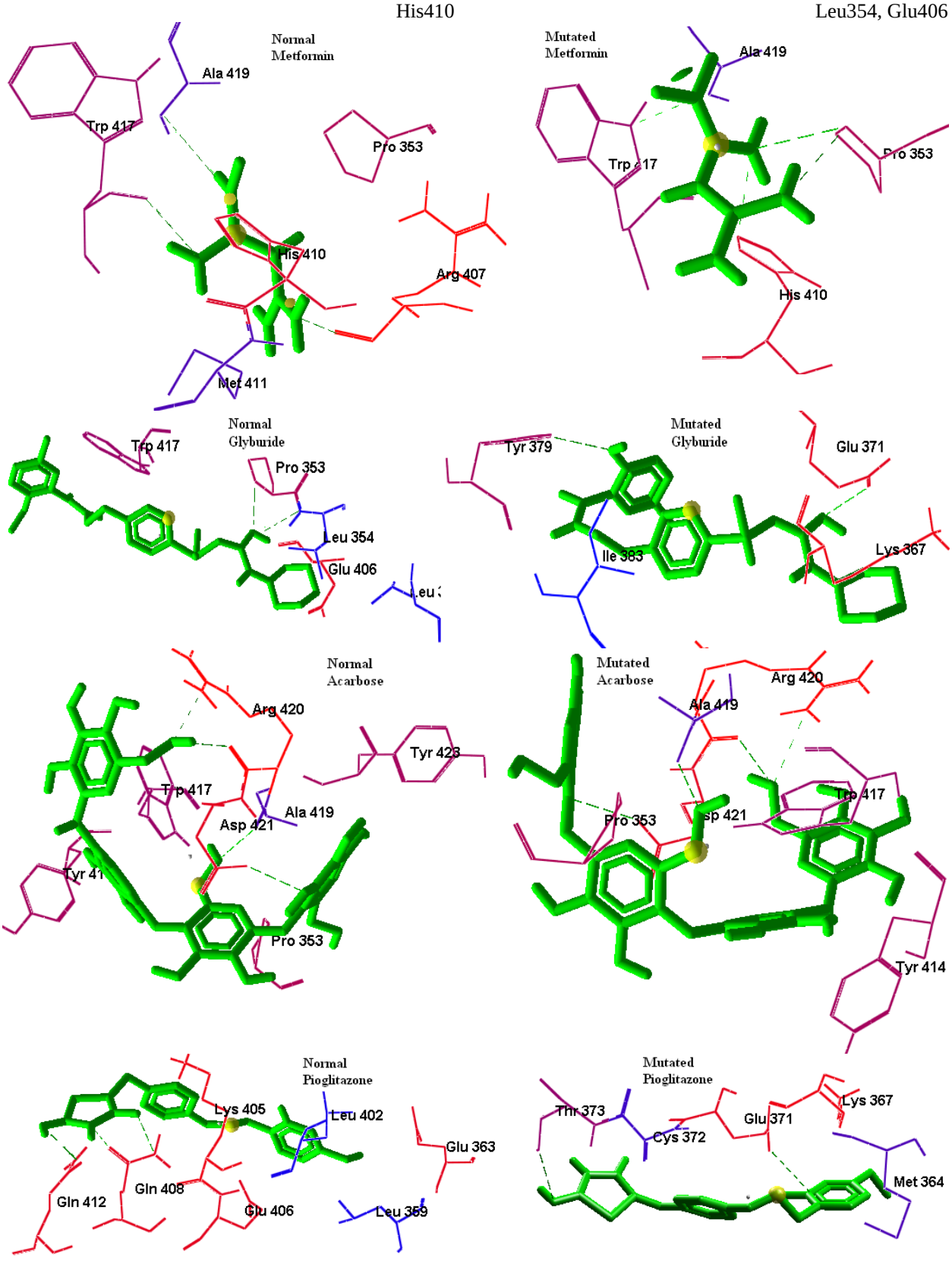
Acarbose	4	-4.15	7.04	Glu406, Arg420, Asp421	4	-2.76	18.23	Glu406, Arg420, Asp421
Pioglitazone	3	-2.55	5.23	Gln408, Gln412	2	-2.37	4.82	Glu371, Tyr373
Voglibose	3	-4.68	14.32	Pro353, Glu406, His410	3	-2.33	7.56	Pro353, Leu354, Glu406



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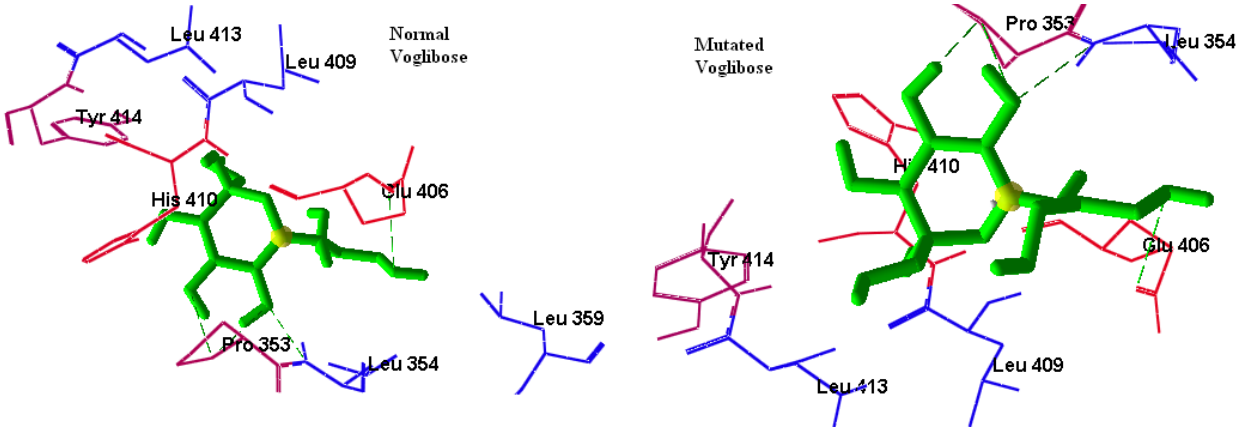

Figure: 4d Molecular docking of normal and mutated TCF7L2 protein structures with anti-diabetic drugs.

e). The APM1 protein has genetic mutation at Arg112cys region in Indian population, the active site amino acids is predicted based on number of pockets and amino acids. The protein has 22 pockets of which 10 amino acids within the surface region; the molecular docking is performed with normal protein within active site amino acids Gly107, Val110, Arg112, Thr121, Tyr122, Thr124, Arg131, Thr133, Gln139 and His142 of binding energy -2.25, -2.22, -2.41, -2.48 and -2.43 kcal/mol respective is forming 3, 5, 7 and 11 hydrogen bonds. The mutated protein is docked with active site amino acids Pro104, Val110, Try111, Cys112, Glu120, Thr121, Tyr122, Arg131, Thr133, Tyr 137, Gln139, Asn 141, His142, Tyr143, Asn153 and Thr243 of strong binding energy of -6.1, -3.16, -2.5, -3.387 and -6.298 kcal/mol and 3, 5, 6, 11 and 14 hydrogen bonds is formed within active site amino acids.

Table: 4e. Molecular docking of normal and mutated APM1 protein structures with anti-diabetic drugs

Ligand	Normal Protein				Mutated Protein			
	No. of H Bonds	Binding Energy	Ki (□M)	Amino acids	No. of H Bonds	Binding Energy	Ki	Amino Acids
Metformin	5	-2.25	60.86	Thr133, Arg131, Thr121	6	-6.1	63.16	Tyr143, Tyr 137, Asn 141
Glyburide	5	-2.22	13.46	His142, Arg112	5	-3.16	91.49	Asn153, Thr243, Try111
Acarbose	11	-2.41	18.1	Gly107, Gln139, Arg112, Gln139, Val110	14	-2.5	108.85	Val110, Cys112, His142, Gln139, Pro104
Pioglitazone	3	-2.48	80.13	His142, Arg112	3	-3.387	75.89	Tyr122, Arg131,
Voglibose	7	-2.43	71.11	Thr133, Arg131, Thr121, Tyr122, Thr124,	11	-6.298	89.88	Thr133, Glu120, Thr121, Arg131, Thr133

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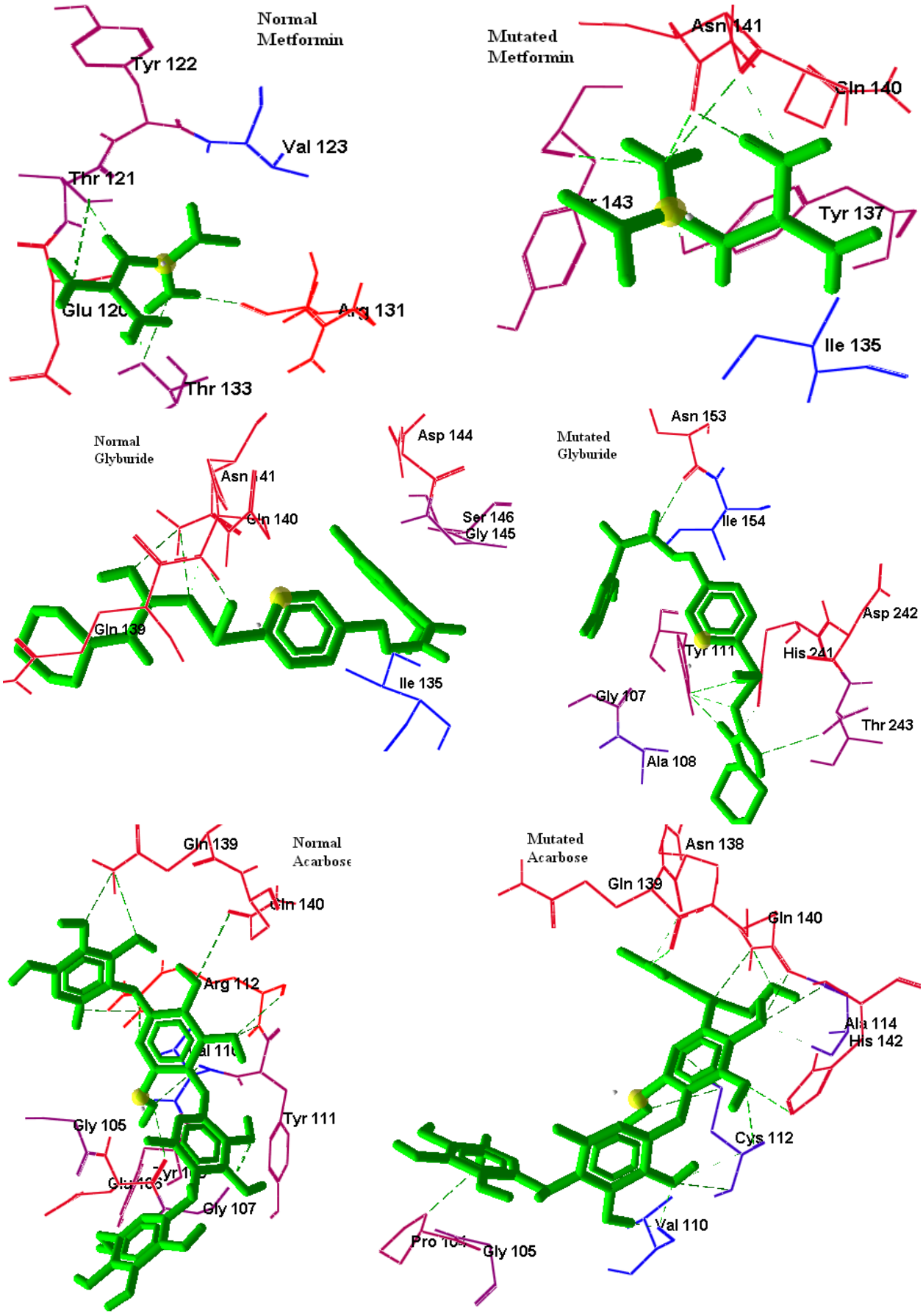

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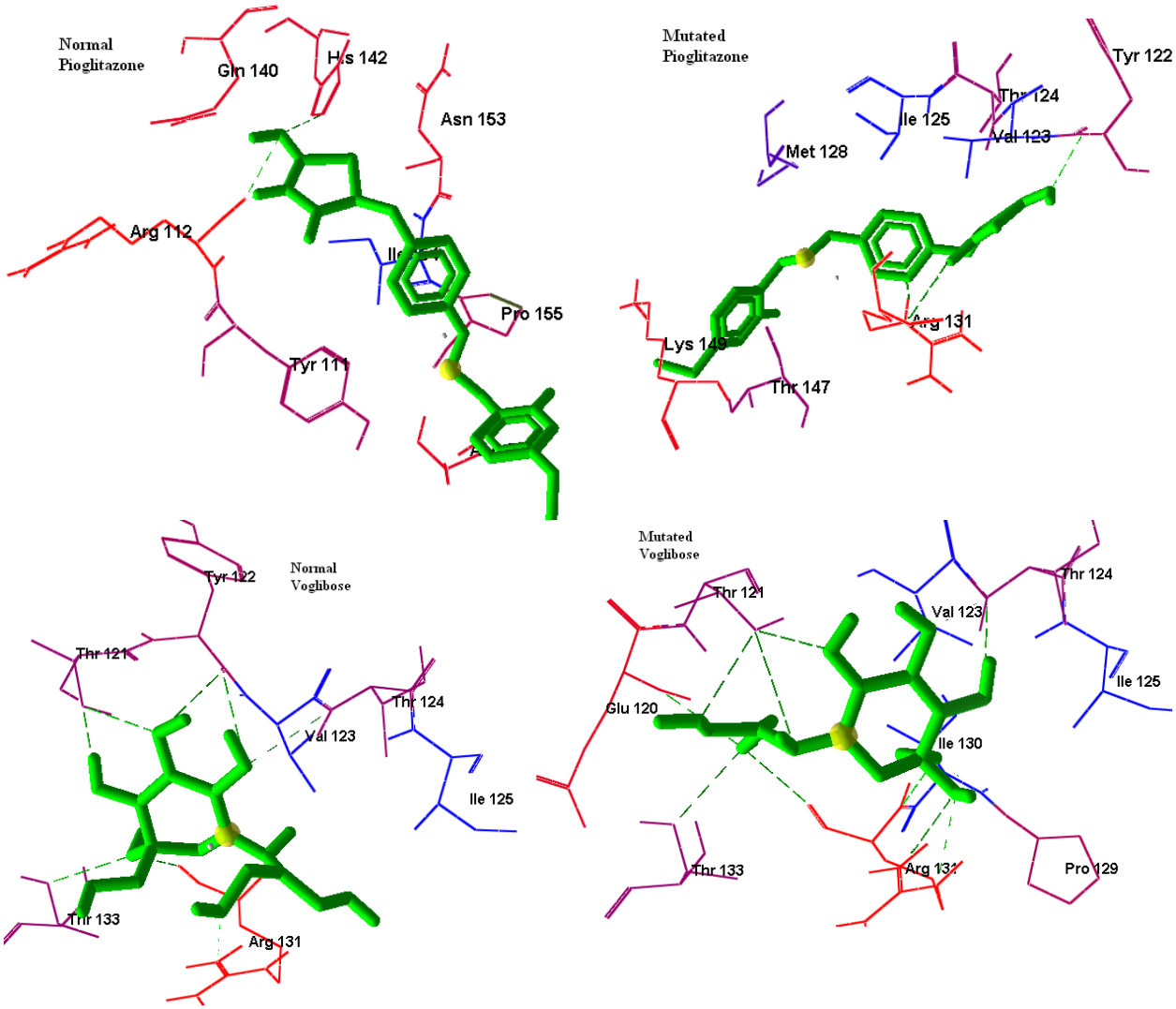

Figure: 4e Molecular docking of normal and mutated APM1 protein structures with anti-diabetic drugs.

Similar study was done by Hernandez et al (2015) to identify the best phenolic compounds from different fruits that helps HNF1A gene to increase the transcription of many proteins which results in controlling the glucose level diabetic patients. In this study anthocyanin delphidinidine-3 arabinoside, flavonide flavone compounds of different berries and citrus fruits. The outcomes of docking study discovered that Anthocyanins at dimerization domain and transcription domain had -8.3 kcal/mol and -7.2 kcal/mol binding energy respectively and Flavonide at dimerization domain had -6.5 kcal/mol binding energy. So the study concluded that because good binding energy compared to Flavonides, Anthacyanins are effective to increase the transcription factor of HNF1A.

4. CONCLUSION

Pharmacogenetic and Pharmacogenomic properties are studied for the novel genetic variations identified in the genes HNF1A, HNF1B, PPARG, TCF7L2 and APM1 which are involved in causing hyperglycemia specially in population of India with respect to the drug compounds such as Metformin, Glyburide, Acarbose, Pioglitazone and Voglibose. For each of the normal and mutated protein structures, docking studies were carried out against the drug compounds to evaluate the efficiency of drug on the pathogenesis of type 2 diabetes mellitus.

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The overall docking study confers that the drug responses of Metformin, Glyburide and Pioglitazone are more effective for all five genes by forming 2-8 hydrogen bonds with good binding energy. The drug Voglibose appears to show adverse effects for two genes HNF1A and APM1 since it has formed 13 and 11 hydrogen bonds respectively. The drug Acarbose has formed 14 hydrogen bonds with APM1 gene, so it is predicted that this drug may show side effects in some cases. The study confirms that the prior knowledge of patients' genetic makeup and medications (Pharmacogenomics) will help to increase the efficiency of therapeutic prescriptions and also shows cost effectiveness in way. Further clinical trials with larger sample size are needed to give increased outcome and cost effectiveness before this promise can be delivered to clinical practice.

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CONFLICTS OF INTEREST

All authors are accepted for publication, there is no conflict of interest.

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VAM FUNGAL ASSOCIATION WITH *PUNICA GRANATUM* IN ORGANIC PLOT IN TUMKUR REGION OF SOUTHERN INDIA

Renuka Ravinath[1], Anupam J Das[1] , B. R. Shilpa[1], N Ramesh[1], Sushil Kumar Middha[2*]
[1] Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore
[2]Department of Biotechnology, Maharani Lakshmi Ammani College for Women, Bangalore

ABSTRACT: Microbial communities play a significant role in soil fertility. In the present study, we report Vesicular Arbuscular Mycorrhiza (VAM) infection in the roots of *Punica granatum.* Standard staining methods followed by microscopic observation was employed to study root samples of *Punica granatum,* collected from an organic plot located in Tumkur region of Karnataka in Southern India. The presence of VAM infection in pomegranate roots were correlated with the soil characteristics. The pH of the soil was found to be highly alkaline (8.6) aside of a rich micronutrient status of the rhizosphere soil in terms of Copper (Cu) (25.9%), Manganese (Mn) (6.84%) and Zinc (Zn) (23.6%). Furthermore, the phosphorous (P) and potassium (K) content in the rhizosphere soil was found to be higher (0.0057% and 0.107%) compared to the control sample away from the root zone (0.0047% and 0.009% respectively). Elevated nitrogen (N) was found in the rhizosphere soil sample (0.191%) in comparison with the control (0.164%). The fruits were found to be bigger, healthy, disease-free and of superior quality compared to the conventional farming plot. The fruits had glossy texture with more number of seeds per fruit, fleshy aril, juicy, sweet in taste and highly pigmented, which could be attributed to the symbiotic association of VAM with the roots of pomegranate. In line with this finding and prior reports, the artificial inoculation of VAM as a biofertilizer can substantially improve the plant growth and productivity in agricultural fields.

Key Words: Biofertilizer; *Punica granatum*; Rhizosphere soil; VAM

Corresponding author*: Dr. Sushil Kumar Middha, PhD
Department of Biotechnology, Maharani Lakshmi Ammani College for Women, Bangalore
Email address: drsushilmiddha@gmail.com

1. INTRODUCTION

The major global challenge of food security for feeding the growing population can be addressed by enhancing the soil fertility and crop productivity in a sustainable manner. Macdonald and Singh have reported that 50-60% enhancement in crop productivity can be ensured by improving the soil health along with the plant related traits [1]. Crop productivity is governed by various factors such as soil fertility in terms of physico-chemical properties of soil [2], climatic factors and more importantly, the soil microbial load. Soil microorganisms play an integral role in enhancing the soil fertility and crop productivity, especially in low nutrient input agricultural fields [3]. These microbes include beneficial bacteria, actinomycetes, fungal associates etc.

In most of the agricultural fields, the limiting nutrient factors are found to be nitrogen and phosphorous, leading to poor plant growth. Beneficial soil microbes facilitate the supply of these nutrients through biogeochemical cycle or by release of these nutrients from insolubl
e form to readily available form to the plants.
In this regard, the symbiotic association between the plant roots and mycorrhizal fungi could be considered as the most beneficial association for crop growth [4]. The availability of phosphorous to plants is limited as they are fixed in acidic and alkaline soil. Plants take up

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phosphorus as $H_2PO_4^-$ or HPO_4^-[5]. The contribution of soil bacteria in chelating the cations through organic acids released by them and releasing phosphorous has not been very encouraging [6]. Mycorrhizal association with crop plants ensure effective absorption of phosphorous and nitrogen from greater soil depth due to the extension of the root system. In return, plant transport the photosynthates through roots which are utilized by the fungi [7]. Vesicular Arbuscular Mycorrhizal fungi (VAM)members belong to the order Glomales. The association is found with the roots of higher plants. VAM infection facilitate the availability of phosphorous and other immobile micronutrients like zinc, copper, boron and molybdenum [8, 9]. Apart from increasing the nutrient availability, VAM also confers resistance to drought, salinity and biotic stress [10]. The plants having VAM association show higher tolerance to both root borne and soil borne pathogens. The widespread VAM infection in many host plants confirms the lack of specificity with respect to host association.

The arbuscular mycorrhizae has the potential to modify the root exudates in the rhizosphere, favoring the growth of beneficial microbiota through its symbiotic association with the host plant [11, 12]. As per the reports on influence of farming practices on mycorrhizal growth, the soil characteristics prevailing in sustainable agricultural fields contribute to better growth of the fungus than the conditions in conventional fields, where there is indiscriminate use of chemical fertilizers, pesticides and tillage practices [8, 13-15]. Mycorrhizal biofertilizer application has gained momentum in the last decade due to its immense potential for improving plant nutrition and plant health. In Indian agricultural scenario, the application of mycorrhizal fertilizer would go a long way in reclaiming the waste land and in afforestation programs.

The current research work carried out in Department of Biotechnology, REVA University was an attempt to explore the microbial diversity in the organic field where pomegranate variety, Bhagwa (*Punica granatum*) was grown as the main crop.

2. MATERIALS AND METHODS

The roots of pomegranate were collected from pomegranate variety Bhagwa, grown in organic plot located at Tumkur, Bangalore. The standard staining method was adopted to observe the infection in the roots [16]. The root segments of 0.5 to 1.5 cm were subjected to alkali hydrolysis with 10% KOH heated at 90^{O}c. Roots were washed with distilled water followed by heating them in 1% HCl for an hour. The acid neutralized roots were stained with 0.05% trypan blue by heating the roots with the stain for two hours. The roots segments were observed under the microscope (40X) for the presence of VAM infection.

3. RESULT AND DISCUSSION

The observed roots showed clear infection of VAM in the cortical cells of the root with prominent Vesicles and Arbuscules (Figure 1). Vesicles represent the storage organs of VAM. Arbuscules are the modified hyphae of the fungus for exchange of the nutrients between fungi and the host plant cell.

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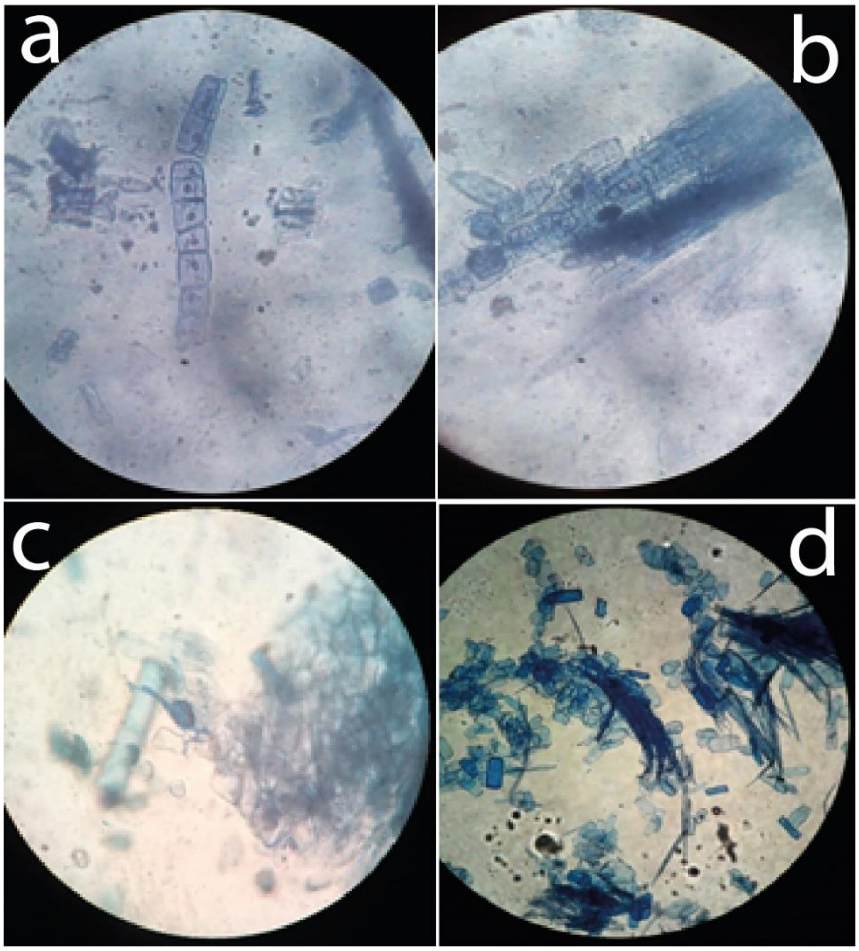

Figure 1. Microscopic examination of *Punica granatum* root samples. (**a, b**) Pomegranate cortical root cells showing VAM infection. (Fungal structures stained dark blue with trpan blue); (**c**) Pomegranate cortical cells infected with VAM, Vesicles and arbuscules clearly seen; (**d**) Pomegranate cells showing heavy infection of VAM Hyphae.

The presence of VAM infection in pomegranate roots were correlated with the soil characteristics. The pH of the soil was found to be highly alkaline (8.6). Well drained soil, the semi-arid climate and the alkaline pH were conducive for the survival of VAM spores and subsequent infection in the root and its establishment. The rich micronutrient status of the rhizosphere as shown in **Table 1** soil in terms of Cu (25.9%), Mn (6.84%) and Zn (23.6%) could be attributed to the symbiotic association of VAM with the roots of pomegranate. The phosphorous content in the rhizosphere soil was found to be higher (0.0057%) compared to the control sample away from the root zone (0.0047%).

Table 1. Chemical characteristics of rhizosphere soil

S No.	Soil Parameter	Value
1	pH	8.6
2	N	0.191%
3	P	0.005%
4	K	0.107%
5	Cu	25.9%
6	Mn	6.84%
7	Zn	23.6%

The potassium content was also higher in the rhizosphere soil (.107%) compared to control

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(0.0093%). The nitrogen status was also enhanced in the rhizosphere soil sample (0.191%) in comparison with the control (0.164%).

The fruits of the plants were of bigger size compared to the conventional farming plot. The fruits had glossy texture with more number of seeds per fruit, fleshy aril, juicy, sweet in taste and highly pigmented.

There is literature evidence that the micronutrient status and N, P, K status of the soil is enhanced where there is symbiotic association established between the host and the Mycorrhiza. This could be attributed to the solubilization of insoluble macro and micronutrients mediated by mycorrhiza which in turn promote the rhizosphere microbial growth.

The artificial inoculation of VAM as a biofertiliser can substantially improve the plant growth and productivity in agricultural fields. Kim *et al.*,1997, have reported an increase in phosphorous content in tomato plants treated with VAM fungus *Glomus etunicatum* [17]. There are also reports of enhanced nitrogen and phosphorous uptake with the synergistic effects of VAM and beneficial soil bacteria. Further studies on quantification of the VAM association with respect to the mycelial sheaths on the roots, the number of vesicles in the infected roots and spores in soil would throw more light on the contribution of this symbiotic association in promoting plant growth and productivity.

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IDENTIFICATION OF EST DERIVED MARKER ASSOCIATED WITH AAI_LTSS SUPERFAMILY FOR PLANT DEFENSE RESPONSE IN *SOLANUM MELONGENA*

Reena Rosy Thomas[1] and M K Chandra Prakash[1]
[1]Division of Social Sciences & Training, Indian Institute of Horticultural Research, Bengaluru

ABSTRACT: Biotic stress in plants affected by pathogen attack involves physiological changes that affects the growth and productivity. To overcome such stresses, identification of markers for development of superior crop varieties tolerant to biotic stress is essential. Brinjal (*Solanum melongena* L.) is an important vegetable crop species, also a good source of minerals and vitamins. EST collections of *Solanum melongena* are having abundant repeat motifs, most of them are hexa repeats. Mathematical algorithm based motif identifier was used to analyze around one lakh EST sequences of *S.melongena* and identified potential markers associated with AAI_LTSS superfamily, a protein family that includes alpha-amylase inhibitors, lipid transfer proteins, seed storage proteins. These protein superfamily are known to play primary role in protecting plants from pathogen, insects, lipid transport between membranes and nutrient storage. Nine EST sequences associated with plant defense response that belongs to AAI_LTSS superfamily have been identified and aligned with MSA. The alignment showed conserved marker region with repeat motif pattern that occurs several times inside the sequence. Identification of such potential marker sequences would be useful for plant breeders in developing biotic stress tolerant crops.

KEYWORDS: AAI_LTSS, Biotic stress, EST, plant defense, *Solanum melongena*

Corresponding Author: Dr. Reena Rosy Thomas
[1]Senior Scientist,Division of Social Sciences & Training, ICAR-IIHR, Bengaluru 560089
Email Address: reenart@hotmail.com

1. INTRODUCTION

Simple Sequence Repeats (SSR) or microsatellites have attracted relatively more attention because of their abundance in plants genome, reproducibility, and co-dominant inheritance [4]. Expressed Sequence Tags (ESTs) analysis is an economical method for identifying microsatellites. As EST derived markers are from the expressed portion of the genome sequences, they are genetically involved with a trait of interest and it could be all or part of the gene directly affecting the trait [9]. As gene-coding segments are found to be conserved among related species, EST–SSRs is expected to have greater transferability between them [5]. As most of EST-derived microsatellites are from conserved coding sequences, they represent functional markers found in the coding regions of the genome [7]. Identifying potential markers located in a specific coding regions in plants continues to be a challenging concern. In this context, identification of markers associated with different stress tolerant genes using *in silico* methods can be used in plant breeding programs for the development of multiple stress tolerant crops. Current research was carried out with an objective to identify stress responsive genes in *Solanum melongena***.** Brinjal ***(****Solanum melongena*L). is an important vegetable crop which has high nutritional value, but is attacked by many pathogens in all growth stages thus limiting the production.

2. MATERIALS AND METHODS

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The EST collections of *Solanum melongena* were downloaded from the NCBI site in FASTA format for identifying the repeat motifs using the freely available repeat masker**,** Tandem repeat finder programs. Non- redundant EST sequences were filtered and analyzed for repeat regions. The ESTs having repeat motif were clustered into groups and analyzed for conserved regions. The sequences having conserved regions along with their IDs are subjected to Conserved domain database (CDD) search engine of NCBI. The output generated from NCBI protein database search and the conserved domains were matched against the respective EST sequences having microsatellite markers,which were categorized by their respective protein superfamilies.

3. RESULTS AND DISCUSSION

EST collections of *Solanum melongena* are having abundant repeat motifs and among the identified ESTs having perfect repeat sequences, hexa motif repeats were dominated followed by tri repeats [8]. The non-redundant ESTs of *S. melongena* having microsatellite markers identified using *in silico* methods associated with AAI_LTSS superfamily are listed below (Table-1) along with EST length, number of similar proteins identified in other species, motif pattern repeated inside the marker sequence. Further, these EST sequences were aligned using multiple sequence alignment (Fig.-1) for its conserved marker regions inside the potential EST sequences having 43 bp, 34 bp and 57 bp shown in Fig.1a, Fig.1b, Fig.1c, respectively.

Table-1: ESTs of *Solanum melongena* having microsatellite markers associated with AAI_LTSS superfamily

Brinjal-EST Ids	EST length	Matches with published proteins	Repeat Motif	EST derived Marker	Conserved region length
FS009203	504	3	$(AAGCCG)_3$	CCTAAACCAAAGCCGAAGCCGAAGCCTAAGCCAACCCCAAGCC	43bp (Fig.1a)
FS009249	511	3	$(AAGCCG)_3$	CCTAAACCAAAGCCGAAGCCGAAGCCTAAGCCAACCCCAAGCC	
FS009592	509	1	$(TTTCC)_2$	TCTTTTCCTTTGCTTTAACCTTTTCTTTGCCTTA	34bp (Fig.1b)
FS023573	615	1	$(TTTCC)_2$	TCTTTTCCTTTGCTTTAACCTTTTCTTTGCCTTA	
FS026358	587	1	$(TTTCC)_2$	TCTTTTCCTTTGCTTTAACCTTTTCTTTGCCTTA	
FS030083	603	1	$(TTTCC)_2$	TCTTTTCCTTTGCTTTAACCTTTTCTTTGCCTTA	
FS038862	476	1	$(TTTCC)_2$	TCTTTTCCTTTGCTTTAACCTTTTCTTTGCCTTA	
FS044799	321	1	$(GAAT)_2$	CAGTTGCTATTAGCATCATTCTCAACAATTGCGGAATGAATAATTCTGGCTTCACTT	57bp (Fig.1c)
AB032755	555	1	$(GAAT)_2$	CAGTTGCTATTAGCATCATTCTCAACAATTGCGGAATGAATAATTCTGGCTTCACTT	

Nine EST sequences (FS009203, FS009249, FS009592, FS023573, FS026358, FS030083, FS038862, FS044799 and AB032755) belongs to Alpha amylase inhibitor - lipid transfer-seed storage (AAI_LTSS) protein superfamily (Table-1). AAI_LTSS are a group of highly-conserved proteins having around 9kDa seen in higher plant tissues [1]. Proteins in this family are known to play primary roles including defending plants from pathogens and insects, lipid transport between intracellular membranes and nutrient storage [3], [6], [2].

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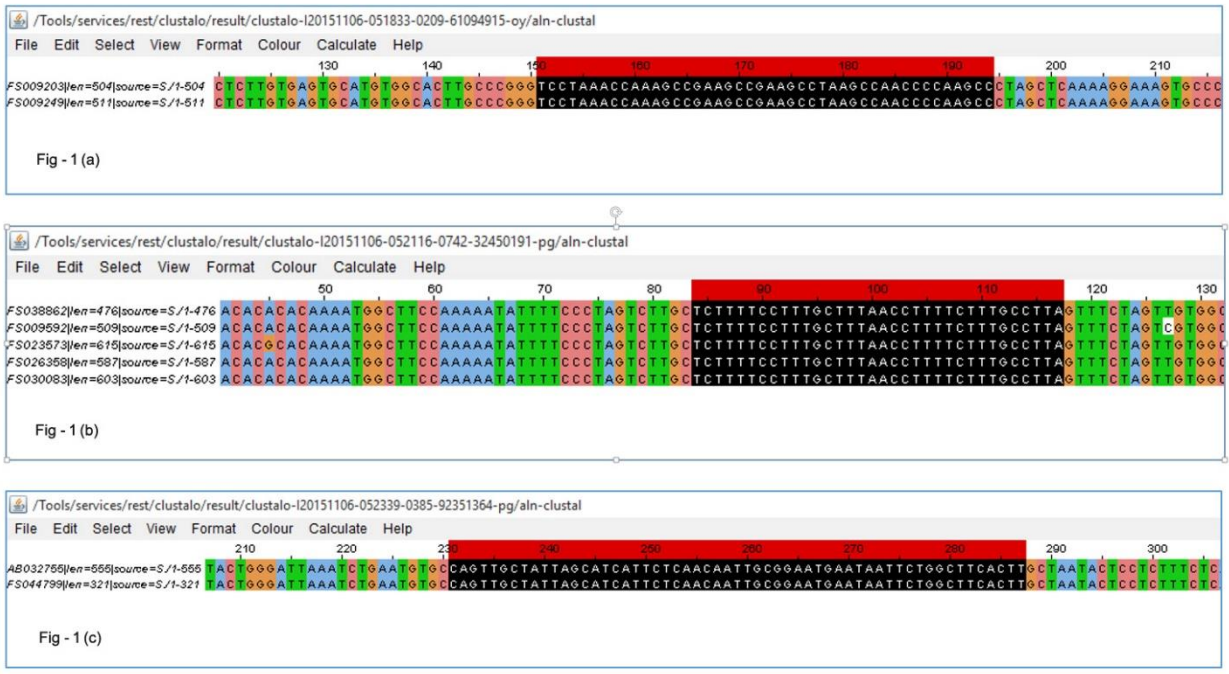

Fig 1. EST sequences aligned with MSA which shows conserved marker region repeated inside the marker sequences.

These EST sequences are highly conserved in several Solanaceae crops and plays an effective role in plant defense response. These unique EST sequences are having conserved domains and has a marker in respective EST sequences. These EST linked markers of AAI_LTSS superfamily serve as potential genetic markers useful in marker assisted selection for identifying the biotic stress tolerant parental lines in plant breeding programs. Therefore, present study provides basic information about plant defense responsive gene lined markers in *S. melongena* that can be exploited in overcoming various pathogen attack problems affecting the crop and limiting the production. Further, it could be useful in marker assisted breeding programs for development of superior crop varieties which yields better under biotic stress conditions.

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INVESTIGATION ON MICROPROPAGATION, ANTIMICROBIAL PROPERTIES & PHYTOCHEMICAL ANALYSIS OF *COSCINIUM FENESTRATUM:* AN ENDANGERED FLORA

Renuka Ravinath[1]*, Sumedha Dahal [1], Priyanka Mishra[1], Sachin Rathod[1], Sabnam Sharma[1], Sonika Rai[1]

[1]Dept. of Biotechnology, School of Applied Sciences, REVA University, Bangalore

ABSTRACT: *Coscinium fenestratum* locally known as Maramanjal is an endangered medicinal plant having diverse pharmacotherapeutic applications for the treatment of diabetes, head and neck cancer, colorectal cancer. It is a plant listed in IUCN Red data book. Since, there are many obstacles with the conventional propagation methods such as 4-5 months germination time and duration of 15 years for the plant to become reproductive, the present study on micropropagation of *Coscinium fenestratum* was carried out with various explants and Growth regulator combinations. The study revealed that for direct regeneration the ideal explant was found to be shoot tip with 20µM/l Kinetin + 0.25µM/l 2,4-D & 20µM/1 BAP + 0.25 µM/1 2,4-D and for indirect regeneration, petiole with 6µM//l 2,4-D+ 0.5µM/1 BAP. The antimicrobial studies with the plant's extract against test organisms showed the highest activity for methanolic extract from the leaves with a MIC of 2.6cm against *Bacillus subtilis.* The present study could standardize the protocol for micropropagation of *Coscinium fenestratum* which would facilitate mass multiplication of the species when scaled up.

Keywords*:* Micropropagation, Kinetin, BAP (Benzyl Amino Purine), 2,4-D (2,4-Dichloro phenoxy acetic acid), *Coscinium fenestratum*, explant, Antimicrobial Properties.

***Corresponding author:** Renuka Ravinath
Associate Professor, Dept. of Biotechnology, School of Applied Sciences, REVA University, Bangalore, Email address: renukamadhu@reva.edu.in

1. INTRODUCTION

Coscinium fenestratum belonging to Menispermeaceae family, is one of the most important plants used extensively in traditional systems of medicine [1]. This medicinal plant has been found to be native to Sri Lanka and the Western Ghats in India [12]. It is used in the treatment of cancer in the head or neck regions such as pharynx, nasal and oral cavity [6, 10 and 13]. Stem pieces are effectively used against diseases such as rheumatism, skin related disorders and jaundice [11]. *Coscinium fenestratum* also finds the application in the synthesis of silver nanorods [4].Phytochemicals such as Alkaloids, Flavonoids, Glycosides, Saponins, Tannins, and Sterols have been reported as the major phytoconstituents of stem extract of *Coscinium fenestratum* [8]. Because of overexploitation and low rate of germination of seeds associated with *Coscinium,* a population reduction of more than 80% over a period of three generation is reported and is on the verge of being extinct. [12]. Vegetative propagation is very slow and conventional propagation through seeds and vegetative cutting are not adequate to meet the demand of conservation and sustainable utilization. Rapid multiplication of plants and further improvement of trees can be achieved through tissue culture of plants and genetic transformation in a limited period of time [3]. Therefore, the development of an in vitro protocol for production of planting material is important to conserve this valuable endangered species.The studies on micropropagation of the plant reported were of preliminary nature and more studies with detailed refinements of techniques were required. Research on *C. fenestratum* has confirmed the in vitro callus induction for determination of alkaloid

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berberinecontent [9] and the later was found to be effective against human Fibrosarcoma cells [7]. Hence, in the present investigation carried out on *Coscinium fenestratum,* an efficient and reproducible procedure for clonal multiplication of this pharmaceutically important plant was carried out. Efforts were also made to qualitatively analyze the phytochemical constituents and antimicrobial properties of this medicinal plant, *Coscinium fenestatum*.

2. MATERIALS AND METHODS

2.1 Plant Collection and Sterilization of Explants

The plants for micropropagation were obtained from IAIM (Institute of Ayurveda and Integrative Medicine), Yelahanka, Bengaluru, Karnataka, India. Plant Material was collected from healthy potted plants. Different explants materials were used for the study. Single nodal regions, mature, shoot tips and petioles, and as well as immature leaf were used. Explants of respective size (table no.1) were excised and transferred into full strength MS media supplemented with different combinations of growth regulator concentrations (table no.2). Single node and shoot tips explants were excised from tender shoots as well as matured shoots of about 5 year old plant of *Coscinium fenestratum* to study the influence of maturity of tissue on explants establishment and plant regeneration. The explants were washed thoroughly under running tap water for 20 minutes which was followed by wash with distilled water containing detergent, Tween-20 for 15 minutes followed by washing with 3-4 changes of distilled water. The explants were then subjected to surface sterilization with 70% ethanol for 30 seconds in LAF followed by surface sterilization with 0.1% $HgCl_2$ for 3 minutes. Surface sterilized explants were washed with several changes of sterile distilled water. All the cultures were then kept in the culture room at 25±1°C with the photoperiod of 16 hours light and 8 hours dark for direct regeneration and complete darkness for initial few days for callusing. The effects of auxins and cytokinins on callus induction and shoot development were evaluated. For each treatment, three replicates were prepared.

Table 1: Explants used

S.No	Explant	Size
1.	Shoot tips	1 cm
2.	Single Node	1 cm
3.	Petiole	1 cm
4.	Leaf	4mm × 4mm

Fig 1. Single node explants, Leaf explants, Shoot tip explants, Petiole explant

2.1.1 Use of Fungicide

Two different concentrations of fungicides i.e., 0.01% and 0.1% were used for sterilization of the explants prior to inoculation to standardize the explant sterilization protocol.

2.1.2 Use of Ascorbic Acid

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To overcome the problem of browning, two approaches were tried using ascorbic acid as an antioxidant. In the first case, 0.1% of ascorbic acid was incorporated in the media. The second approach was to have a combination of media supplementation and treatment of explants with 0.1% ascorbic acid after fungicide treatment during surface sterilization.

2.2 Preparation of Medium

Full strength MS medium was used as the basal medium in all the cultures supplemented with different auxin or cytokinin combinations for direct and indirect regeneration.

Table 2: Explants and Growth Regulator Combinations

For Indirect regeneration

Sl. No.		Treatments	Explants used
1	C1	1µM /l 2,4-D + 0.5 µM /l BAP	Leaf and Petiole
2	C2	2µM /l 2,4-D + 0.5 µM /l BAP	Leaf and Petiole
3	C3	3µM /l 2,4-D + 0.5 µM /l BAP	Leaf and Petiole
4	C4	6µM /l 2,4-D + 0.5 µM /l BAP	Leaf and Petiole
5	C5	8µM /l 2,4-D + 0.5 µM /l BAP	Leaf and Petiole
6	C6	10µM /l 2,4-D+0.5 µM /l BAP	Leaf and Petiole

For Direct Regeneration

SI.No		Treatments	Explants used
1	T1	10µM /l Kinetin+ 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf
2	T2	20µM /l Kinetin+ 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf
3	T3	30µM /l Kinetin+ 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf
4	T4	10µM /l BAP + 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf
5	T5	20µM /l BAP + 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf
6	T6	30µM /l BAP + 0.25 µM /l 2,4-D	Single Node, Shoot tips, Petiole & leaf

2.3 Antimicrobial Studies

Both aqueous and methanolic extracts of the leaves of *Coscinium fenestratum* were used in 75:25 & 50:50 dilutions to study the antimicrobial properties on different plant and animal pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *E.coli using* inhibition zone method [8,9]. Streptomycin was used as standard antimicrobial agent at the concentration 20mg/100ml as a positive control. Negative control plates containing sterile distilled water were also prepared.

Fig 2. Methanolic Extract from leaf and Aqueous Extract from leaf

2.4 Minimum Inhibitory Concentration

Both the methanolic and aqueous extracts were concentrated by removing the solvent by the

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use of Rotary Vacuum Evaporator. The fine powder obtained was used to prepare a stock concentration of 100µg/ml from which dilutions of 7µg/ml, 10µg/ml, 25µg/ml and 40µg/ml were prepared. Similar procedure as 2.5.2 was followed using the working concentrations of plant extract (7µg/ml, 10µg/ml, 25µg/ml and 40µg/ml) prepared from stock solution of concentrated extract.

2.5 Phytochemical Analysis

2.5.1 Phytochemical screening by chemical method

The screening of extracts of *Coscinium fenestratum* was carried out for the confirmation of phytochemicals such as tannins (gelatin test), flavonoids (NaOH and Pew's test), Alkaloids (Mayer's test, Iodine and Wagner's test), Saponins (Froth test), Sterols (Salkowski test), Glycosides (Keller killiani test) and Phenolic compounds (Ellagic test and phenol test).

3. RESULTS

3.1 Standardization of sterilization procedure for efficient culture establishment

With the general sterilization procedure, the percentage of contamination for leaf, single node, shoot tip and petioles as explants was found to be 100%.The complete control of fungal contamination was given by 0.1% of Bavistin and the aseptic culture percentage was found to be 100%. None of the explants survived though the aseptic cultures were established by fungicide treatment, due to browning of explants.The combination of media supplementation and treatment of explants with 0.1% ascorbic acid after fungicide treatment during surface sterilization were found to be effective in preventing the browning.

3.2 Studies on Interaction Effects of Growth Regulator Concentrations and Explant Type for Direct Regeneration:

The success rate in direct regeneration was satisfactory. Single node was found to be the ideal explant for multiple shoot formation of *Coscinium fenestratum.* The ideal explants and growth regulator combination for direct regeneration was found to be shoot tip with growth regulator combinations of 20µM/l Kinetin + 0.25µM/l 2,4-D (T2) & 20µM/l BAP + 0.25µM/l 2,4-D(T5).

Table 3: Effect of growth regulators on the survival percentage of explants for direct regeneration

TREATMENTS	EXPLANTS				MEAN
	Leaf	Shoot tip	Petiole	Single node	
Basal	0	0	0	14.28	3.57
T1	0	0	0	28.57	7.14
T2	0	14.28	0	42.86	14.29
T3	0	0	0	14.28	3.57
T4	0	0	0	28.57	7.14
T5	0	14.28	0	28.57	10.71
T6	0	0	0	0	0
MEAN	0	4.08	0	22.45	6.64
For comparison of means		F			CD @5%
Explant (E)		16.01			S

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Growth regulators (G)	1.83	NS

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	553.9009	6	92.31681	1.838487	0.147843	2.661305
Columns	2412.188	3	804.0627	16.01288	2.54E-05	3.159908
Error	903.8427	18	50.21348			
$Total	3869.932	27				

From the analysis of the ANOVA table, each treatment's performance is not very significant from the other treatments since F< $F_{critical}$ and P> 0.05 whereas each explant's performance is highly significant from the other since the F > $F_{critical}$ and P> 0.05.

Fig 3: Sprouting from single node and shoot tip respectively

Table 4: Number of multiple shoots per explant of *C.fenestratum* as influenced by explant and growth regulator concentrations

Treatments	Explants		
	Shoot tip	Single node	Mean
Basal	0	0	0
T1	0	0	0
T2	3	2	2.5
T3	0	0	0
T4	0	1	0.5
T5	2	1	1.5
T6	0	0	0
MEAN	0.72	0.57	0.64

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	11.71429	6	1.952381	8.2	0.010839	4.283866
Columns	0.071429	1	0.071429	0.3	0.603645	5.987378
Error	1.428571	6	0.238095			
Total	13.21429	13				

From the ANOVA analysis, it was observed that F>F critical for the treatments used, hence it has significant role in number of shoots developed from the explants $F_{critical}$ for whereas, for the explants used since F<$F_{critical}$ so, it does not have significant role.

Table 5: Effect of growth regulators for callus induction on the survival percentage of explants

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TREATMENTS	EXPLANTS		MEAN
	Leaf	Petiole	
Basal	0	0	0
C1	0	0	0
C2	0	0	0
C3	50	33.33	41.67
C4	100	100	100
C5	0	33.33	10.71
C6	0	0	0
MEAN	21.43	23.8	22.62

For comparison of mean F CD @5%Explant (E) 0.17 NS Growth regulators (G) 25.01

ANOVA

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	16864.99	6	2810.832	25.00134	0.000537	4.283866
Columns	19.8254	1	19.8254	0.17634	0.68916	5.987378
Error	674.5635	6	112.4272			
Total	17559.38	13				

From the analysis of the ANOVA table, each explant's performance is not very significant from the other treatments since $F < F_{critical}$ and $P > 0.05$ whereas each treatment's performance is highly significant from the other since the $F > F_{critical}$ and $P < 0.05$.

Fig 4. Plates with stages of callus induction from petiole

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3.3 Antimicrobial Studies

3.3.1 Antimicrobial studies of the methanolic leaf extracts in various concentrations on the test organisms:

Fig 5. Plates of *P.aeruginosa, S.aureus, E.coli* and *B.subtilis* showing inhibitory zone diameter.

Table 6: Effect of the various dilutions of methanolic leaf extracts on test organisms.

S.No	Extract type	Mean Inhibition zone diameter (cm)			
		P. aeruginosa	*S.aureus*	*E.coli*	*B.subtilis*
1.	Positive control	2.4	3.1	3.4	3.8
2.	Crude	-	-	3.3	3.4
3.	75:25	-	-	3.05	3.65
4.	50:50	-	-	3	3.5
5.	Negative control	-	-	-	-

Among the methanolic leaf extracts of *C. fenestratum*, all the extracts had similar inhibitory activity against *B.subtilis* and *E.coli* whereas no inhibition was observed against *P. aeruginosa* and *S.aureus.*

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	29.54125	3	9.847083	18.37238	0.000354	3.862548
Columns	6.9075	3	2.3025	4.295932	0.038588	3.862548
Error	4.82375	9	0.535972			
Total	41.2725	15				

Since F> $F_{critical}$ in both the cases, hence the test organisms and dilutions used both have significant role in determining the minimum zone of inhibition.

3.3.2 Antimicrobial Studies of the aqueous leaf extracts in various concentrations on the particular organisms:

The aqueous extracts in the crude and diluted form (75:25 and 50:50) had no inhibitory

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activity against all the test organisms (*B.subtilis, E.coli, P.aeruginosa* and *S. aureus*).

Fig 6: Plates showing no inhibition against *B.subtilis, P.aeruginosa, S.aureus* and *E.coli*

3.4 Minimum Inhibitory Concentration (MIC)

Table 7: MIC of the methanolic leaf extracts in various concentrations on the test organisms

S.No	Extract type	Mean Inhibition zone diameter (cm)			
		P. aeruginosa	*S.aureus*	*E.coli*	*B.subtilis*
1.	Positive control	2.4	3.1	3.4	3.8
2.	7µg/ml	-	-	-	-
3.	10µg/ml	1.6	1.3	1.3	2.6
4.	25µg/ml	1.8	1.4	1.9	2.85
5.	40µg/ml	2.35	1.9	2.7	3.4
6.	Negative control	-	-	-	-

The MIC of the methanolic plant extract was found to be at 10µg/ml for all the test organisms. Maximum inhibition zone diameter (2.6cm) was observed for *B.subtilis* whereas the zone diameter for *E.coli and S.aureus* was 1.3cm.

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	3.003	3	1.001	7.91174	0.003547	3.490295
Columns	23.02175	4	5.755438	45.49004	3.72E-07	3.259167
Error	1.51825	12	0.126521			
Total	27.543	19				

From the analysis of ANNOVA table we can see that the different test organism as well as the

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concentrations are very significant towards the diameter of zone of inhibition as $F>F_{crit}$.

Table 8: MIC of the aqueous leaf extracts in various concentrations on the test organisms

S.No	Extract type	Mean Inhibition zone diameter (cm)			
		P. aeruginosa	*S.aureus*	*E.coli*	*B.subtilis*
1.	Positive control	2.3	3.2	3.55	3.8
2.	7µg/ml	-	-	-	-
3.	10µg/ml	-	-	-	-
4.	25µg/ml	3	3.4	2.2	2.6
5.	40µg/ml	3.5	3.4	2.7	2.8
6.	Negative control	-	-	-	-

The MIC of the aqueous leaf extract for all test organisms was found to be 25µg/ml. The maximum inhibitory zone diameter (3.4cm) was observed with the *S.aureus* whereas less inhibitory zone diameter (2.2cm) was observed with *E.coli*.

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.266375	3	0.088792	0.458181	0.71652	3.490295
Columns	44.6505	4	11.16263	57.60116	9.87E-08	3.259167
Error	2.3255	12	0.193792			
Total	47.24238	19				

From the analysis of ANOVA, we can observe that since $F> F_{critical}$ for concentrations used, hence concentration has significant effect on MIC whereas $F< F_{critical}$ for test organisms, so that have no much significant effect.

3.4 Phytochemical Analysis

3.4.1 Phytochemical screening

The qualitative tests performed on the extracts revealed that alkaloids, flavonoids, saponins, glycosides, sterols, triterpenoids and phenols are present in the extract whereas tannins are absent.

4. DISCUSSION

Explant of *Coscinium fenestratum* exudes phenolic compounds into the media which is oxidised by polyphenol oxidases leading to browning and death of explants. Similar obstacles in micropropagation were reported [2] during tissue culture of *T.cordifolia*, which was overcome by antioxidant treatment. The use of ascorbic acid in the culture media proved to be better solution to overcome the browning and also greatly increased the number of plantlets produced [15]. Thus, the final explant sterilization was modified by treating the explant with bavistin for fungal contamination and explant treatment with 0.1% ascorbic acid as well as media incorporation of the same to control browning. The use of 0.05% ascorbic acid in the culture media proved to be better solution to overcome the browning and also greatly increased the number of plantlets produced The best treatment which induced shoot formation in minimum duration from shoot tip and single node explants was found to be (T2) 20µM/l Kinetin and 0.25µM/l 2, 4-D followed by (T5) 20µM/l BAP along with 0.25µM/l 2,

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4-D with a mean value of 14.29 and 10.71 respectively. *C. fenestratum* stem extract showed the most effective activity against *Neisseria gonorrhoeae* [6].In the present study, both shoot tips and single node explants took almost equal number of days to exhibit shoot initiation. This may be because both the explants are derived from almost the same part of the plant having similar physiological and ontogenic status. The presence of phytochemicals such as alkaloids, flavonoids, saponins, glycosides, sterols, triterpenoids and phenols were also confirmed.The maximum zone of inhibition was observed against *B.subtilis* (3.65cm) followed *by E.coli* (3.05cm) with the 75:25 dilution extract among the three extracts used. In the study of MIC, it was found that the MIC for methanolic extract was found to be 10µg/ml whereas for the aqueous extract was found to be 25µg/ml showing the antimicrobial properties of phytochemicals present, especially the phenolic and flavonoid constituents.

5. CONCLUSION

The current investigation reports on standardizing the protocol for micropropagation which included selection of best explants, selection of the right concentrations of growth regulators and growth media. The investigation also reports the antimicrobial property of the plant extracts, where the methanolic extract showed the highest activity. Thus, the micropropagation of this endangered plant can ensure mass multiplication and continuous supply of plant material for pharmaceutical purposes. Further studies on secondary metabolite production from callus can go a long way in conserving this medicinal plant.

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DISTINCTIVE APPLICATION OF VARIOUS COMPUTATIONAL APPROACHES TOWARDS REMEDIATION OF POLLUTANTS PERSISTING IN MARINE ECOSYSTEM

Avinash Sangram Belure[1], Rachana Arvind[1], Jaladurgam Ramya Teja[1], Vishal Chanda[1], Prabhakar Mishra[1]*

[1]Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore, Karnataka-64

ABSTRACT: Marine pollution has been a major concern in day to day scenario which is growing with the modernisation of the population. The persistent contaminants imminent to the nautical bio-network are majorly due to anthropogenic activities. The toxic elements like organic pollutants (insecticides, herbicides, polychlorobenzene, Bisphenol –A) while inorganic contaminants like heavy metals are the prime cause behind this hazardous issue. Majority of these effluents sedimenting at the lagoons and coral reefs are causing a threat to the marine inhabitants resulting in depletion to marine flora and fauna. Rising toxicity of marine ecosystem is the matter of apprehension as it upsets the non-target species causing deterioration of marine diversity. Therefore, there is an urge to combat the problem of marine pollution through a combination of life science study with the computational world such as Particle Tracking Method, numerical model and metagenomic applications. The current study explains the beneficial impact of computational study in context to bioremediation of prime pollutants persistent in the ecosystem, which in turn can become an efficient remedy for this dangerous problem.

Keywords: Marine ecosystem, Organic Pollutants, Inorganic Pollutants, Computational Approach

***Corresponding Author:** Dr. Prabhakar Mishra
Assistant Professor, Department of Biotechnology, School of Applied Sciences
REVA University.E-mail: prabhakarmishra@reva.edu.in ; prabhakar.science99@gmail.com

1. INTRODUCTION

Marine environment inhabits definite areas within the ocean. Few are productive nearshore regions, comprising of salt marshes, mangrove forests and estuaries. Remaining appear to be absolutely barren, like the ocean floor. The hydrosphere joins all freshwater and saltwater systems. The elevated salt content (salinity) and global flow make marine ecosystems varied from other aquatic ecosystems. Additional physical factors that govern the dissemination of marine ecosystems are temperature, geology, tides, geography and light availability [1]. Marine ecosystems contain abyssal plain like deep-sea coral, whale fall, brine pool, Arctic, Antarctic, deep sea (abyssal water column), coral reef, hydrothermal vent, kelp forest, mangrove, open ocean, rocky shore, salt marsh and mudflat, and sandy shore. Some of these regions are very dynamic. Remaining are in endless darkness where photosynthesis is not possible. Few marine ecosystems undergo extreme deviations in physicochemical parameters like oxygen levels, temperature, light availability and other factors on a regular basis. Others are legitimately stable and exhibit slight change at varied seasons [2]. The creatures that dwell into the marine ecosystems are as unlike as the ecosystems within themselves. They must be highly adapted to the physical conditions of the ecosystems in which they live.

Open Ocean is the biggest marine ecosystem. It comprises of almost 65 per cent of the volume entire world ocean. The open ocean zone usually denotes to the upper 656 ft. of water. This differentiates it from the deep sea ecosystem below. This ecosystem is highly

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diverse and dynamic attaining diverse forms of life. The diversity of within the open ocean living forms ranges from whales, dolphin, sea turtles and megafauna, or large animals like sharks to minuscule plankton and small fish species. , as well as large migratory fish, also displays an important portion in this diverse and extensive ecosystem. Although the megafauna is large and tend to show the dominance, the invertebrate species in actual terms dominate the ecosystem with over 95% of total population in this ecosystem. Various plankton population drift along on ocean currents, leading to the formation of food web base. These open ocean currents transmit nutrients to various parts of the ocean. Currents also help few animals towards the migration which leads to a distribution of their eggs throughout the ocean. Even though the abiotic scenario like salinity, circulation, light, and temperature of the open ocean are impartially consistent, even then there are particular areas where the life is plentiful and sparse.

Coral Reefs predominantly occur all through the subtropical and warm tropical regions of the ocean. Some corals survive in deeper and colder waters. Corals can be categorised as hard and soft corals. These corals are made up of minute creature from an animal kingdom called polyps. Few corals comprise of a single polyp but the majority stay in a colonial manner. Colonial corals comprehend millions of polyps clubbed together. Few of the examples for soft and flexible corals include the sea plumes sea fans and sea whips. True and soft corals embrace mushroom, tree and black corals. On the stony, reef-building, corals generate the calcium carbonate skeletons and are accountable for making the structure of the reef. Few of the common stony corals consist of plate corals, pillar corals, brain corals, and branching corals like staghorn and elkhorn. The coral polyps responsible for reef formation tends to divide and forms forming layer upon layer upon their growth. These corals tend to have a symbiotic relationship with the algae termed as zooxanthellae which prevail within the tissues of the reef-forming corals. Coral reefs are one of the furthermost naturally diverse ecosystems in the world and are home to the variety of living forms which ranges from sponges, jellies to octopus, sharks and manta rays. They also deliver feeding, depositing, nursery, and refuge areas for numerous marine species. Corals are brittle systems and their growth and health can be dependent on various features like elevated temperatures, decreased water clarity, wave action from storms and nutrient runoff.

Mangrove ecosystems involve woody mangrove trees and shrubs with the salt-tolerance feature. They are found in shallow, sandy or muddy areas with low oxygen content along shorelines. About 80 diverse species of mangrove trees are found all through the tropical and subtropical regions. The Black and white mangroves are low salt-tolerant in comparison to the red mangrove and are found beyond from the water's edge. They have distinct adaptations to aid them towards obtaining the oxygen and discharge of the excess salt. Red mangroves have the utmost salt-tolerance property and are established adjoining to the shores. They are habitually submerged in the shallow water with a dense, moderately bare network of roots termed as prop roots which nurture down from their branches. The roots of these mangroves aid to several essential features. They alleviate the shoreline by gripping the wave action and reducing the water flow. This results in the accumulation of sediments which prevents surplus sediment and nutrients from reaching adjacent coral reef and seagrass ecosystems. The prop roots also support as a substrate for attachment for various species of tunicates, sponges, shellfish and algae. This mangrove forest delivers a multifarious habitat for numerous ecologically and economically vital organisms, which includes sponges, barnacles, snails, mussels, algae, birds and fishes. Majority of these living forms utilize the mangrove as a source of feeding and protection. This tends to form these mangrove ecosystems a vibrant part of sustaining fisheries inside and in line to mangroves. These mangroves once concealed large areas of coastlines, but in the current scenario due to the coastal development and increase in the pollution has led to substantial depletion of various

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mangrove habitat throughout the world.

The mentioned ecosystem is the hub for various flora and fauna which tends to get interlinked to make a marine environment. Currently, due to the increasing anthropogenic activities and human intervention, the threat towards this vast marine ecosystem is being observed. Various forms of chemical pollutant [3,4,5] which are categorised as organic and inorganic derived chemicals are the key element towards this havoc [2]. The contamination arising due to these xenobiotic components results in depletion of various forms of ecosystem prevailing in this environment [3]. The current review, therefore, summarises about the different forms of chemically derived pollutants, their toxic impact on the marine environment and the application of different computational strategies opted to remediate the pollutants from their profound place of contamination, making the remediation approach to become a potent tool towards the issue to marine pollution.

2. ECO-TOXICITY OF XENOBIOTIC COMPONENTS TOWARDS MARINE ENVIRONMENT

The threat of eco-toxicity generating from the involvement of chemical pollutants to the marine environment is a major concern being dealt with in the current scenario. These chemically derived pollutants tend to have their origin from various sources like industrial effluents, agricultural waste and waste from the anthropogenic activities leading to contamination towards the various ecosystem prevailing in the marine environment. In general, the pollution that culminates in the marine bodies gets derived from distinct sources (Figure 1). Majority of these pollutants tends to originate from land mass either in form of runoffs or discharges that accounts to 44 % of the total xenobiotic compound. 33 % of the marine contamination through xenobiotic component is from the atmosphere. The other 22 % of marine pollution is from shipping accidents, maritime and sewage or garbage dumping. Lastly, the offshore mining process corresponds to 1% respectively. The borough and industrialised runoff, in combination with the agricultural effluents, comprise of elevated levels of elements such as phosphorus and nitrogen. These two elements play a key role towards the plant growth but are habitually persisting on the marine ecosystem in leading to plentiful organismal growth. A continuous influx of water comprising of these nutrients arising from the land consequently disturbs the balance of the ecosystem. As there is an elevation in the concentration of nitrogen and phosphorous, the microalgae population tends to rise resulting in the algal colossal bloom [6]. When these algae die, the left out remains leads to consumption of oxygen persisting in the marine ecosystem making the system more anoxic and therefore plays a role in mortality of aquatic species.

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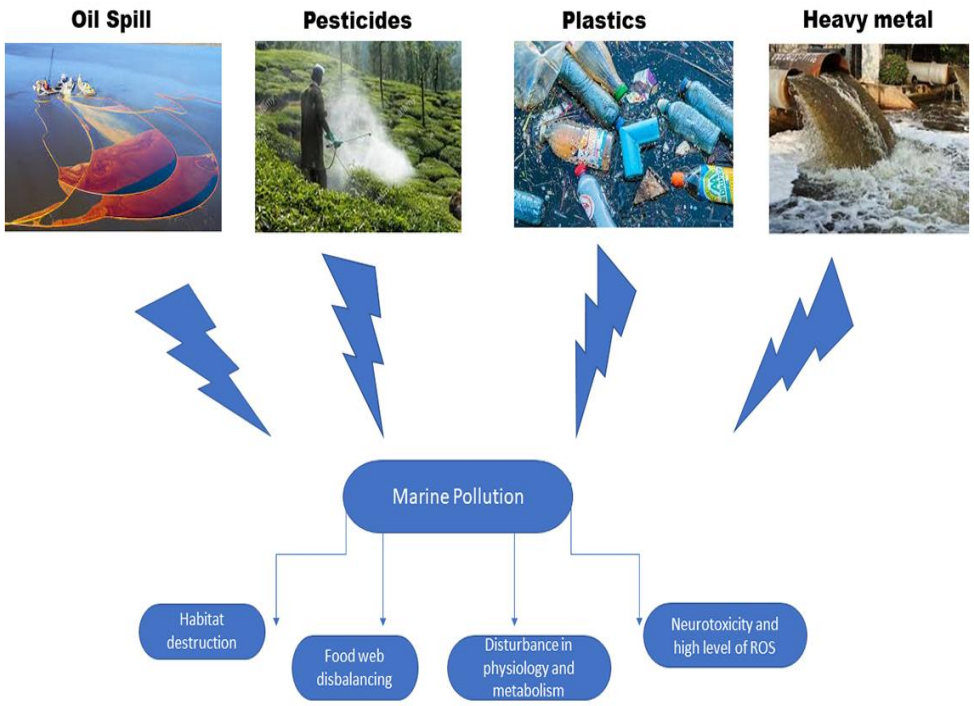

Figure 1: Sources of marine pollution and their ill impact on marine ecosystem

The chemical pollutants leading to marine contamination can be categorised into two basic forms i.e. organic and inorganic pollutants. The toxic elements like organic pollutants (insecticides, herbicides, polychlorobenzene, Bisphenol –A) while inorganic contaminants like heavy metals are the prime cause behind this hazardous issue. The hazardous effect of the interactive pollution from cadmium and mercury towards marine gastropod was studied by Lavie and Nevo 1986 [5]. Similarly, Ansari et al., 2004 described the heavy metals pollutants as natural elements of the marine and freshwater environment, usually found in minimal concentrations [7]. Anthropogenic activities have unavoidably resulted in the elevation of metal ions in numerous water bodies including the marine environment. Other sources which lead to the pollution of heavy metals as pollutants include offshore oil and gas exploration, Mine drainage, domestic effluents, agricultural runoff, acid rain and industrial runoff. The excessive dumping of these hazardous waste to the marine system results in the increased metal concentration which later gets incorporated into the aquatic sediments [8,9,10]. The accumulation of these pollutants in the marine ecosystem results as lethal agents producing toxicity on aquatic organisms. These toxicity implications due to heavy metals and hydrocarbons are categorised as a direct and indirect effect. The indirect effect is through a disturbance in the food chain which dis-balances in tropical transfer and results in ecological stress. The direct effects are seen in behaviour, migration, physiology, metabolism, reproduction, development and growth of aquatic animals.

The furthermost lethal pollutants present in the marine environment are the plastics. The tiny sized derivatives of these plastics are in forms of microplastics and nano plastics. In an aquatic ecosystem, these nano-sized plastics are readily ingested by numerous forms of biota related to the aquatic environment, either by false perception as prey or fouled by planktonic prey materials [11]. Plenty of organisms containing fur seals (Arctocephalus spp.), , harbor seals (*Phoca vitulina*), brown shrimps (*Crangon crangon*), Humboldt squids (Dosidicus gigas), copepod (*Tigriopus japonicus*), gooseneck barnacle (Lepas spp.), lugworms(*Arenicola marina*), Norway lobster (*Nephrops norvegicus*), amphipods (*Orchestia gammarellus*), shore crabs (*Carcinus maenas*), oysters (*Crassostrea virginica*), various fish

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(cod, whiting, haddock), birds, turtles, blue mussels (*Mytilus edulis*) [12]. The pollutant and their ingestion into the bodies of marine flora and fauna make the toxicity to accumulate into the body of these organisms. This, in turn, makes these tiny molecules a noxious pollutant [13,14].

Another form of marine pollutant are the oil spills. These oil spills generally originate from the shipping accidents and then also dumping of waste from ship harbours [15]. Ship's engines in addition to the burning of garbage results in the production of greenhouse and harmful gases like derivatives of carbon, sulphur and nitrogen which in-turn leads to the formation of acid rain and global warming, which is harmful to the marine organism. The harmful hydrocarbon dissociating from the oil enters into the aquatic system through food changes making the disturbance in the food chain and lethality in the aquatic species [16].

Apart from the plastics, oil spills and land runoff in form of heavy metals, the pesticides tend to behave as a potent pollutant towards marine pollution. The various forms of pesticides like organophosphorus, organochlorines, and pyrethroids tend to shoe their lethal impact as a marine pollution source. The main source of pesticide contamination to the marine environment is through the agricultural runoff. The uncontrolled application of these conventional pesticides, which are hydro-immiscible, gets emulsified using the organic solvents. The application of these organic solvents to pesticides results in the solubilisation to water in-turn leads to an increase in the toxicity [17]. The pesticide prevailig in the environment act as immune-modulators which makes the changes in the humoral and cellular immunity of aquatic species. These pesticides tend to show their action as neurotoxic depleting the choline esterase activity, while elevation in ROS (Reactive oxygen species) upon generation of toxicity in the body. The deep-sea mining is yet another source of marine pollution where the toxicants released the mining pose a substantial threat towards the marine environment [18]. Therefore, the mentioned pollutants (Table 1) play a major role as leading to disturbance in the flora and fauna of the mine ecosystem, this type of contamination not only effects the organism but also tends to show its lethal impact towards humans through entering into the food web.

Pollutant type	**Hazardous effect**	**REFERENCES**
Industrial Effluent	Anoxic condition in the marine ecosystem	Ryther 1971
Heavy metals	Disturbance in food web and reproduction, development and growth of aquatic animals.	Dallinger et al., 1987
Microplastics	Abnormal and lethal changes in the physiology, metabolism due to increased stress and ROS increase	Cole et al., 2011; Andardy 2011
Insecticides	Disturbance in the Sodium Pump, Neuro toxicity, Paralysis	Antizar-Ladislao 2008; Yamazuki 2011
Oil Spills	Anoxic condition; disturbance in the food chain and tropical transfer	Jordan and Payne 1980; Thompson and Eglinton 1979

Table 1. Various forms of the pollutants persisting in the marine ecosystem and their lethal impact

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3. COMPUTATIONAL APPROACH TOWARDS REMEDIATION OF TOXIC CHEMICALS IN MARINE ENVIRONMENT

3.1 Remediation of Heavy Metal by Computational Approach

The process of remediation is not sufficient in today's world of rapidly accumulating pollutants so, computational tools and models are being incorporated with biological entities for further advancement of the remediation technique which will only result in non-toxic products and hence, reducing toxic elements from the environment. One such contaminant is heavy metal which remains persistent in any ecosystem and is a threat in the marine ecosystem.

Bioremediation of heavy metals includes Physico-chemical methods and Biological methods. Physiochemical methods include Chemical precipitation, Oxidation or reduction, Filtration, Ion exchange, Reverse osmosis, Membrane technology, and Electro-chemical treatment. These methods are ineffective at low concentrations of heavy metals (<100mg/l). Water-soluble Heavy metals also cannot be separated by Physico-chemical methods. Biological methods of Bioremediation is mainly mediated with Microorganisms which can transform Heavy metals from one oxidative state to another oxidative state or one organic complex to another, Nitrogen fixation gene nifH reduces N_2 to NH^{4+}, N to $NH^{4+}$is more efficient in the accumulation of Arsenic by *P. vittata*. and have the capability of dissolving metals [19]. Microbial remediation depends upon the association of microbes to heavy metal either independently or through mechanical stress [20].

The toxicity of heavy metals gets reduced by the action of microbes on the functional groups i.e., the microbes inhibits the functional group and it results in inactivation of its toxicity. Microbes will also modify the active conformations and the displacement of essential ions to inactivate its toxicity. Effect of microbial action depends upon the concentration of heavy metal, availability of heavy metal, Type of metal, Nature of medium and the species. Many contaminants of heavy metals Inorganic contaminants like Arsenic, Chromium and Cadmium disrupts the cell membrane of some microbes, but cells may develop a defence mechanism including the formation of an outer cell membrane. This protective material is often hydrophobic involving chemiosmotic ion pumps which won't allow toxic heavy metals to pass through the membrane. Hence, showing resistance to heavy metal (As, Cr, and Cd) contamination. Aspergillus, Penicillium, Rhizopus are potential microbial agents for the removal of heavy metals.

The process of bioremediation through microorganisms is mainly through Biosorption, Bioaccumulation, Siderophore formation, Biosurfactants production. The other process that involves the process of bioremediation includes Biomineralization, Biotransformation and bioleaching. Biosorption is defined as the process in which the cell wall of microbes which is rich in polysaccharide, lipids and protein will bind to functional groups (Carboxylate, Hydroxyl, Amino and Phosphate) of heavy metals to perform its action. The rate of reaction depends upon the composition and chemical kinetics on cellular surface, on microbial total biomass and geochemistry of system. It is a passive metabolism (do not require energy). The study of Biosorption includes Genomics, Next Generation Sequencing and genome editing techniques [21,22]. They are mainly used to study organisms with respect to potential biosorption capacity. Bioaccumulation is an active metabolic process (requires energy) which undergoes when the rate of absorption of contaminant is higher than losing. It describes bio-transformational capabilities changing toxic chemicals to non-toxic form to reduce the toxicity of contaminant while keeping it contaminated. The organism Rhodobacter spheroids localized Extracellular (cell envelope) of cobalt, nickel and immobilize R-coo- groups to inhibit its toxic effects [23,24]. The study of bioaccumulation includes Transcriptome analysis which is used to study the roles of important genes in response to heavy metals between different organs in the same organism [25, 26]. Spectroscopy-based proteomic

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technology used to study heavy metal stress at translate level and changes in protein expression due to the accumulation of increase in contamination of toxins [27].

The other process of Bioremediation includes Siderophore formation, it is described as the formation of Siderophore aided by membrane protein-mediated metal transport and formation of Siderophore metal complexes [28]. The study of Bioremediation includes Mass Spectrometry-based Proteomic technology, Proteomic Investigation of Secondary Metabolism (PrISM), Proteo-genomics and bioinformatics analysis, which are mainly used in the study of heavy metals and to design models to reduce its toxic effects [29]. Systems biology is also an important approach to the study of genes, mechanism and signalling pathways during siderophore formation. Biosurfactants production, the precipitation of heavy metals takes place through sorption and desorption at the soil-water heavy metal matrix leading to heavy metal precipitation [28]. The study of this includes Metagenomics which are used to select the best Biosurfactants used for marine ecosystems [30]. Mass spectroscopy-based Proteomics results in the discovery of localization regulators [31]. Biosurf database, this database contains information about biosurfactants and their organisms, protein, metabolic pathways associated Algorithms and Bioinformatics tools.

3.2 Remediation of plastics by Computational Approach

The computational approach has become a core need for study of bioremediation techniques which targets inorganic effluents also, like (halogenated hydrocarbons, xenobiotic compounds, plastic wastes, heavy metals, etc. [32]. Various strategies developed in the past few years have helped in the degradation of many inorganic, persistent pollutants like heavy metals and plastic in the marine ecosystem [33]. Heavy metals as sediment on the floor bed is a harmful effluent to microbes at the beginning which at later stages become harmful for the megafauna and flora present, by bioaccumulation, whereas, plastics are the most persistent and harmful pollutants in today's scenario not only to terrestrial bio-network but to the nautical life as well. Bioremediation of such plastic effluents have become a chief concern from a research perspective and for the general public because of their pertinacious property and baneful effects and fragmentation (mesoplastic and microplastic) like Polycaprolactone a synthetic polymer is degraded by bacterium (*Alcaligenes faecalis*), Polylactic acid degraded by thermophilic bacteria (*Bacillus brevis*) [34].

Bioremediation is playing a crucial role in controlling environmental contamination. Remediation procedures such as chemical physical and microbial are being used to mitigate the persistent pollutants [35]. Microbial degradation of plastic is an eco-friendly approach and doesn’t have an adverse effect to the non-target species, therefore, is being socially accepted method. In recent years some microbes from the Pseudomonas genus were isolated and being used for remediation and plastic degrading [36]. But, the increasing load of the pollutants those microbial properties seem to be inefficient for the work. Applying systems biology helps in obtaining the preliminary data and the metabolic engineering tool is incorporated for enhancing the bioremediating capacity [37]. Integration of system biology and metabolic engineering for bioremediation goes like, primarily the genetic data of the isolated microbial species (Pseudomonas species – Strain: RCL01 & TCL04) is collected (genomics and metagenomics for DNA, Transcriptomics and meta-transcriptomics for mRNA, proteomics and meta-proteomics for protein studies and metabiomics for study of metabolites) and were studied for the bioremediating activity and the amount of plastic which is being degraded by them [38]. As discussed, the genomic studies are done to analyse the amount of plastic disparaged by them, this study is further incorporated with the metabolic engineering and the metabolic pathway of functional genes, proteins, enzymes of some organisms of Streptomyces species, are reconstructed with the help of data repositories [39]. This engineered microorganism is then introduced to contaminants and the degraded product tends to turn into a non-toxic product [37]. Recent studies going on in this field have found

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and confirmed some bacterial (*Pseudomonas stutzeri*) and fungal (*Penicillium simplicissimum*) (for degrading Polyurethane) microbes achieving better results than the previous discoveries [36].

Another method for the quantitative determination of polyacrylamide polymers in water is done using independent component analysis incorporated with Mahalanobis distance method but this method requires incorporation of UV-vis spectroscopy. In the study quantitative determination of polyacrylamide polymer is done, the synthetic polymers collected from the contaminated water and are subjected to classification based on the presence of phthalate polymer using liquid chromatography, then the reverse technique of visual spectroscopy was applied to investigate low- and high-density polymers [40]. In the recent study, FTIR (Fourier Transform Infrared) spectroscopy was applied to the sample, this helped researchers to fetch a detailed molecular arrangement and presence of functional groups [41]. The obtained spectra result from the FTIR is measured for the correlation and coefficient among the different polymer samples by applying Independent Component Analysis which is a computational-mathematical tool [41]. Finally, the results were kept in the tab for engineering the present microbes such as bacteria (*Pseudomonas stutzeri*) and fungus (*Penicillium simplicissium*) and further laboratory study is still under observation [42]

3.3 Remediation of Oil Spills by Computational Approach

An account of an oil spill in the marine ecosystem is of a huge concern and to come up with remediation becomes crucial, an oil spill is a very broad aspect to obtain a single remedy is not an easy option, taking into consideration the spatial distribution of an oceanic region and the amount of oil spill that could be in gallons and hence it's important to bring about computational approach through the introduction of mathematical models termed as Oil spill modelling, which includes a lot of data, mathematical algorithms and numerical [43]. To obtain a required set of data that helps to incorporate and obtain a model for remedy, it's important to plan and make a response framework [44]. So, a note of oil type, spill location, ambient environment conditions, spreading, emulsification, advection, evaporation etc. are repeatedly evaluated to make a series of data sets, based upon which a proper model for remedy can be made for [44]. Many different Oil simulation models have been developed and implemented were based on Coastal management, scenario-based strategies and response strategies and most of these models mainly focused upon the weathering, trajectory of oil, spill response and final fate of oil [45,46]. NRDAM/CMR(Natural Resource Damage Assessment Model for Coastal and Marine Resources) is a comprehensive model introduced by Department of Interior regulation and CERCLA, this model mainly focused upon the biological, chemical and economical damages causing to the marine ecosystem [47] and GNOME developed by National Oceanic and Atmospheric Association is another model that focused upon the trajectory of an oil in water [48]; there were many other models as well based on different goals or scenarios some even included free packages.

An oil spill response is a broad aspect, it could be addressed in various ways, a tactical response is one way which is a plan of an act after a spill has occurred. This focuses on the spill location, recovery of the oil, dispatch of equipment, dispatch origin, optimal equipment and the associated costs [49]. BLOSOM (Blowout and Spill Occurrence Model) is a tactical model which is explicit in the spatial-temporal aspect of an oil spill. This Oil Spill Clean-up model aims at minimizing the costs involved in dispatch time and other associated costs while still meeting the required efficiency in Oil Spill Clean-up [50,51,52,53]. BLOSOM is an Oil Spill simulation program written in C++program, which provides for sudden blowout events and clean up oil spills on the offshores which basically emanate from deep or ultra-deep blowouts [52,54]. When an accidental Blowout occurs in the staging areas,

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they are informed and the equipment vessels at the particular staging areas are provided in terms of random integer 1 to 4 [55]. To minimize the costs of clean-up efforts mainly the distance of the oil spill location is considered because with increasing distance there is increasing costs associated. There are clean-up targets which depend upon the capacity of different vessels that range from 0 to 100% and the BLOSOM simulations estimate the time required to bleach the oil that is discharged due to spillage [55]. The output of BLOSOM simulation is a series of parcels which have a unique identifier and each parcel has a definite set of characteristics that define the ocean conditions, initial start characteristics and weathering. The optimization model is an aspect of BLOSOM which assigns a binary value to uncleaned and cleaned parcels i.e.; 0 and 1 respectively and the obtained information from the model and the unique identifier for a specific parcel, the cleaned parcels are removed from the successive simulation. Another aspect of the BLOSOM simulation is the vector impact grid where the fate of an oil geographically is known, the GIS-derived impact grid gives information about the oil beached and the impact on the coastal lines, the local assets. Most importantly TIG (Total Impact Grid) is a grid where the value for the cells is assigned through oil modifier which is combined with the local assets, which finally tells the impact on the total number of local assets and amount of oil corresponding to a cell. Along with BLOSOM is combined another important model, OSCOM (Oil Spill Clean-up Operation Model) is defined based on a set of parameters like oil clean-up target, operating capacity of equipment, oil spill volume at site, time of dispatch vessel from staging areas, available vessels in number and also decision variables like dispatch from staging area to spill site and based on all the parameters mathematical variations are made to meet constraints like reducing the equipment costs or dispatch time, equipment capacity limitation is invoked at staging areas, the equipment limitations to clean an amount of oil are set, ensuring cleaning of site when the vessels are sent to the containment area and to meet the desired clean-up targets. The model tries to optimally meet the clean-up targets with minimized costs based on the available set of resources which is ultimately a crucial process [55].

Degrading oil spill by microbes through ecological studies and providing their metabolic potential through understanding diversity, functional properties and mass flow among species provide a good insight. Physiological data requirements are accessed from databases [56]. The information on the microbial communities is few due to the complexity and technical limitations [57]. Ecological studies are progressing significantly through metagenomic studies. The bottom-up mathematical framework is cooperative in the advancement of microbial ecological studies by combining physiological data with quantitative methods through framing a model [56]. Understanding the microbial communities through ecological studies are provided from systems biology and inclusion of their individual approaches [58,59]. Oil spill degradation is sequential and the diversity of microbes are affected by an oil spill [60]. Degradation of alkanes is the first by microbes like Alcanivorax species followed by Polycyclic aromatic hydrocarbons (PAH) by cyclo-clasticus. Nitrogen and phosphorous are important in oil degradation and conceptual models exist on its importance, by adding liquid fertilizer enhance biodegradation [60]. Specific microbes degrade different molecules belonging to major classes of oils like PAH, alkanes, nitrogen and phosphorous levels which affect oil degradation [60,61,62,63].

MCA (Metabolic Control Analysis) and HRA (Hierarchical Regulation Analysis) both are Flux Analysis which provides information on cell components and also processes in understanding biochemical changes upon disruption and further analysed for tropic and metabolic interactions among species with their respective abiotic environment [64,65,66]. Flux Balance Analysis (FBA) gives a metabolic network determining the flux of metabolites in the system its properties all obtained from genomic data. FBA also provides information on single species [67]. FBA is done in a steady state environment and dFBA (dynamic Flux

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Balance Analysis) is applied to the dynamic environment, the marine microbial ecosystem being dynamic itself over time and space taken into consideration aids in oil degradation optimization like the growth of Acanivorax after an oil spill [60,68]. Multispecies community-level studies using FBA helps in understanding the microbial interactions are based on different consequences, the bioremediation strategies can be modified [58]. Multispecies FAB approach has been done recently, cell numbers of a single species in microbial interactions are determined [69]. The FAB approach in multispecies has been applied for three species so far [70]. Microbial interactions that are metabolite based are obtained by FBA by incorporating the metagenomic data obtained from computational approach and metabolite network construction [71]. The Limitation of nutrient is essential in oil degradation as it provides interactions among degrading microbes being either competitive or mutualistic and the nutrient uptake is included as a constraint in dFBA obtained by simple Michaelis-Menton equation [72]. Interaction among species at metabolite-based networks and FBA multispecies approach helps in exploiting initial degraders like Alcanivorax and Cycloclasticus to either inhibit or stimulate based on they being antagonistic or synergistic could optimize oil degradation of highly toxic PAH and alkanes [58].

Bioavailability of highly hydrophobic hydrocarbons for microbial degradation is low due to interfacial tension, can be reduced by using surfactants [73]. The optimization of culture conditions aids in better degradation of oil and also by considering the nutrient requirements [74]. Statistical methods are used for optimization like factorial design and response surface analysis. Plackett-Burman(PB)design is used to obtain variables in the oil remediation process that are major important factors and Response Surface Methodology (RSM) along with Box-Behnken design is used to determine interactions among the variables, their effects and the parameters are optimized accordingly [75,76]. PB design is a two-level factorial design, the components of the media that remove oil significantly can be recognized.

$Y= \beta_o + {}^{k}\Sigma\beta_i X_i$

Y - response; β_o- model intercept; β_i – linear coefficient; X_i- code level of independentt variable; k – number of variables involved [77].

The Box-Behnken design was used to obtain the optimum levels for the significant factors that consume oil obtained from PB design, by quadratic model structure and model coefficient true values were determined in 26 trial design matrix for the test bacterium, the most significant variable was selected for determining optimal level [78]. Pseudomonas sp.sp48 showed good efficiency of degradation of oil with supplementary glucose, Yeast extract to seawater media. Addition of surfactant and fertilizer increased the efficiency of oil removal up to 54 to 57% concentration respectively. From statistical analysis 4 variables tested having optimal levels were 0.7418, 0.4, 0.1 g% for glucose, MgSO4.7H2O and Triton X-100 respectively and inoculum size of 4.18ml% giving 100% oil consumption tested on Pseudomonas sp. 48 [74].

3.4 Remediation of Pesticide by Computational Approach

Currently, computational approaches are being a helping hand for remediation of many harmful and persistent pollutants one of them being a pesticide [79]. Computational databases and computer programmes are greatly helping in the development of remediation in terms of physical and chemical degradation, bioremediation in terms of microbial degradation. Its run-down time and much basic laboratory experiments before the application of pesticide degradation, the studies important to predict the possible pathways by using computational tools. Bioremediation is a defence concept that covers individual layers of multi-stage involution in the removal of toxic waste from polluted surroundings. It is generally recognised as the mass-balance-wise most important root of pesticide degradation. A number of micro-organisms capable of degradation of a pesticide include dehalococcoide

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species [80]. This bioremediating technique is not actually removing of the harmful pesticide

instead detoxifying the effects of these effluents like organophosphates, organochlorines and this was initially demonstrated using genetically engineered microorganisms. This detoxification has not been applied on large scale because of its high cost and low degradation rate. The most promising approach for reducing the presence of persistent pollutants in the environment is bioremediation incorporated with computational approaches, genetic engineering and biochemical tests.

Table 2: Different computational approaches for pollutant remediation persisting in marine ecosystem.

Pollutants	Source of Contamination	Computational Approaches	REFERENCES
Heavy Metals (Cadmium, Chromium, Copper, Mercury, Nickel)	• Offshore oil and gas exploitation. • Mine drainage. • Domestic effluents. • Agriculture run offs. • Industrial run off.	• Genomics • Mass Spectrometry-based Proteomic technology • Proteomic Investigation of Secondary Metabolism • System Biology	• Bao et al., 2016 • EI-Metwally et al., 2014 • Leung et al.,2014 • Shi et al.,2015 • Italiano et al.,2009 • Banik et al., 2014 • Chen et al.,2013 • Jackson et al., 2015 • Ciesielska et al., 2014
Plastics (Macro plastic, Mesoplastic, Micro plastic)	• Improper Plastic waste disposal.	• System biology • Metabolic engineering • Genomics • Transcriptomics • Metabiomics • Mahalanobis distance method • Independent Component Analysis (Some research and large scale application still at preliminary stage)	• Dangi et al., 2018 • Sekiguchi et al. 2011 • Calabia et al.,2004 • Dangi et al., 2018 • Fossi et al.,2012 • Lambert et al.,2016
Pesticides (Halogenated hydrocarbons, Xenobiotics Compound:- (Organophosphorous, Organochlorin	• Agricultural land runs off. •	• Transcriptomics. (Research & Large Scale Application Still under progress)	• Lovely.,2013

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e) Eg:- DDT.			
Oil Spills (crude oil and fuel oil) Components: Alkanes and monocyclic/ Polycyclic aromatic hydrocarbons	• oil tanker operations and shipping • sea accidents • oil drilling in offshore	• BLOSOM model and OSCUM model • Metabolic Control Analysis. • Hirearchical Regulation Analysis and Dynamic Flux Balance Analysis). • Bottom up mathematical framework in application to systems biology and genomics • Plackett –Burman (PB) design and Box-Behnken design	• Box et al., 1960 • Allison et al., 1993 • Farag et al., 2018 • Feist et al., 2007 • Getz et al., 2003 • Head et al., 2006 • Mohana et al., 2008 • Nelson et al.,2017 • Nelson et al., 2015 • Roling et al., 2014 • Roling et al.,2007 • Sim et al.,2015 • Socolofsky et al., 2015 • Yao et al., 2009 • Zhuang et al., 2011

The significant interest in using microbes for bioremediation is that it is simply cheaper and more environmentally friendly than the more commonly used non-biological options. The beginning of high amount of DNA Sequencing, Analysis of Gene expression and Modelling of Microbial metabolism and revolutionary study of environmental microbiology is done using metabolomics, proteomics, transcriptomics which are the computational approach for metabolic engineering and engineered microorganism like dehalococcoide species. While, the molecular techniques have helped to improve the bioremediation properties, of microbes, investigation of this field in a preliminary stage of the new era which will help the metabolic capability of the microorganism living in the contaminated environment. This is said to be the new genomics era of pesticide bioremediation [81]. These provided computational approaches makes the remediation to become ease to comfort against the hazardous pollutants that prevails in the marine ecosystem. The different computational approach ranging from genomics to transcriptomics, also different
statistical and design of experiment tools leads to enhancement of the activity driven by microflora as a remediating agent. Therefore, these computational approaches (Table 2) which can be utilized for enhancing the remediation process for the hazardous pollutants persisting the marine environment and making the remediation process more reliable and efficacious.

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4. CONCLUSION

The prime contaminants of the marine ecosystem in present era are classified into two major groups, Organic (oil spills, Pesticides) and Inorganic (Xenobiotic compounds, Heavy metals, Plastics), which are a threat to the marine population. These effluents come from various sources such as oil spills from oil tanker wreckage, pesticides and heavy metals from land runoff and plastics from improper sewage disposal. This increases the toxicity in the marine ecology and is detrimental for microfauna as well as megafauna. As it is known that oil is immiscible to water so it creates an insulating layer over the water surface which does not allow the oxygen to diffuse and hence lowering the availability of oxygen for aquatic biome. The increased use of pesticide in this scientific era is another reason by which marine life is getting affected and it does not stop till there as these persistent pesticides' bioaccumulates and reach the higher order organisms in huge concentrations than at the beginning. Heavy metal such as cadmium increases beyond toxic level wills directly affects endocrine system of fishes. The oxidative property of these heavy metals detoriates cell membrane. The plastic contaminant is another major effluent contributing to marine pollution. The macro plastic from improper disposal will reach the marine water bodies and will be a threat to the mage fauna present as those animals may ingest the plastics accidentally; another harmful effect of plastic is that it fragments and accumulates in form of mesoplastic and microplastic and is dreadful to the microbiome system.

Remediation of these effluents is the only way tackling the toxic effects of them, not only the contaminated ecosystem gets affected by them but also non-contaminated rather the contaminants may also have adverse effects on other ecosystems as seen in the case of bioaccumulation. In the early studies of remediation different physical and chemical methods were used which showed a low cost, fast degrading results but the painful side of the same is that it leaves traces of toxic elements or the degraded product still have some negative impact. To overcome this problematic situation, the help of nature was taken, precisely telling the use of microbes was even cheaper and environmentally friendly and the product released was obviously non-toxic, but the only drawback using a microbial culture for remediation (bioremediation) is that the process of degrading by these organisms is too slow. So, a combination of computation models, genetic engineering and consortia of microbes such as bacterial and fungal species were found to be the most promising and accepted technique of contaminant remediation in the new era.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FOLIAR APPLICATION OF NPK, GROWTH AND YIELD ESTIMATION OF MAIZE CROP UNDER MOISTURE STRESS CONDITION

Saket Kumar[1]*, Rajeev Pratap Singh[1] and Rajesh Singh[2]
[1]Environment Science and Technology, Institute of Environment and Sustainable Development, BHU, Varanasi
[2]Department of Genetics and Plant Breeding, Institute of Agricultural Sciences, BHU, Varanasi

***Corresponding author :** Saket Kumar
Environment Science and Technology,
Institute of Environment and Sustainable Development, BHU, Varanasi
E-mail: saketjntradelink@gmail.com

ABSTRACT

Maize (*Zea mays* L.) growth and yield are most sensitive to nutrients applications under moisture stress condition. Improper fertilizer and water management are the two major factors adversely affecting maize growth and productivity under dry land condition. Plant organic matters are control plant metabolism processes, with nitrogen, phosphorus, and potassium being some of the biochemical metabolism. Foliar application of NPK (2%) was applied in various combinations (N, P, K) in 2 split doses (one split at 30 and second 60 days after germination, at the field IESD BHU Varanasi during rabi (winter) season 2016-17. Foliar-application of N seven times more efficient than soil applied. P uptake is enhanced by the availability of soil moisture; it deficiency is mostly visible in young maize crop. P deficiency symptoms in maize include stunted and yellowing leaf margins, after maturing and hard of leaf and grow more slowly but always disappear when plants grow about three feet. The experiment was laid out in randomized complete block design using three replications. Growth and yield estimation of maize under moisture stress condition, to which physiological maturity produced taller plants (219 cm), higher mean single leaf area (419 cm^2), biological yield (10238 kg ha-1), heavier grains (213 g/1000), grain yield (2883 kg ha-1) and harvest index (24.7%) than the control plot. Thus, foliar application of NPK could not only apply nutrients in the maize crops but it could also be providing water to the thirsty crops under moisture stress condition. All development activities were irrespective to nature and magnitude affect measurable in the environment.

KEY WORDS*: Zea mays;* Growth; Yield; foliar application*;* NPK;Phosphorus deficiency.

1. INTRODUCTION

Maize (*Zea mays* L.) growth and yield are most sensitive for nutrients absorption under moisture stress condition. Maize established under unique environments for germplasm adaptation as climate change will be generate in maize development [1]. A proper fertilizers and water managements are major factors, adversely affecting maize growth and productivity under stress condition. Proper use of fertilizers is replaced nutrient deficiencies for maize growth and development [2]. Nutrient deficiencies are often occurs for a maize crop due to survival in adverse environment [3]. Present time maize productivity techniques are involve foliar fertilizer under water stress condition at appropriate time used for increase maize production as for formers profit under dry land condition. Maize response to foliar application of NPK leads to management direct nutrient availability, and balanced use of nitrogen (N), phosphorus (P) and potassium (K) fertilizers could play a pivotal role inincreasing the yields of cereals under

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moisture stress condition [4]. NPK foliar application are increase crop productivity in easy way under moisture stress condition. Such types foliar application of NPK is not only provides the nutrients but can also provide a significant amount of water stress condition (Li, 2007). The use of modern irrigation way particularly manage water loss and maximize crop production from foliar supplements [5].Poor water availability and high temperatures are less significant during critical phases of maize [6], but maize hybrids are survive in different environment and give more yield under stress condition [3]. Grain yields are reduced (22.6-26.4%) from improper supplements of NPK [5] due to loss kernel weight and kernel number in water stress conditions [6]. The aim of my objective to find out a suitable foliar NPK application and its time of application for improving maize crop under Rabi (winter) season and maximizing yield under moisture stress condition.

2. MATERIALS AND METHODS

The field experiment was conducted under water stress condition to foliar NPK combination at the IESD field BHU Varanasi. The experiment was design in randomized block design using three replications of sub-plot size of $3\times3m^2$, having 4rows with 70cm space, with plant to plant space is 30cm. The fertilizers were 150 kg/ha DAP and 200 kg/ha urea during sowing of maize crop [3], foliar application after 30 days and 60 days after germination, as T1 (2%) at 30 days after germination and T2 (2%) at 60 days after germination along with control. The 2kg NPK were dissolve in 100 litter H_2O for foliar treatments, such as F0= control, F1= N, F2= P , F3= K, F4= N + P, F5= N + K, F6= P + K, F7= N + P + K**.** Data were recorded plant height, mean single leaf area, yield and yield components on days to 50% physiological maturity. The plant height data was taken from base to top 5 selected maize plants in each treatment. Data on biological yield was recorded after harvesting, the harvested material was dried up to constant weight and from the spring balance converted into biological yield Harvest index = Grain yield ÷ Biological yield x 100. Data were statistically analyzed [7] and means were compared using LSD test ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Maize crop maturity

NPK combination and interaction had significant effects on maize crop maturity, as control had no significant effects on crop maturity. Among the nutrient combinations, application of N alone delayed the physiological maturity. Application of foliar NPK decreased the vegetative growth period of maize crop and increase physiological maturity of maize crop. Such types maize physiological developments are increased from P application and helped maize life cycle [8].

Table1: Effect of foliar NPK combination and its application time on days to physiological maturity, plants, height, single leaf area and biological yield (kg/ha) of maize under moisture stress condition.

Foliar Nutrient (1.5%)	100 grain weight	Grains Ear	Grain yield (Kg/ha)	Harvest Index (%)
N	234.8	372.4	2657	27.4
P	221.3	378.6	2388	25.9
K	219.8	381.9	2453	23.6
N+P	223.2	376.3	2862	26.8
N+K	221.6	372.8	2537	23.7
P+K	213.7	382.6	2193	21.6

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N+P+K	224.5	366.9	2459	26.3
LSD				
Application Time				
2% at 30 DAG	215.3	383.1	2463	25.6
2% at 60 DAG	227.4	368.5	25.62	25.2
1% each at 30 & 60 DAG	225.2	368.5	2755	23.5
LSD 0.05	1.05	ns	24	ns
Control	201	321	206	23.5
Interaction	*	ns	*	ns

Means in this category fallowed by similar letters are significantly different at 5% probability (0.005).

3.2 Plant height

Statistical analysis of the data indicated that nutrients combination had significant effects on plant height. Combined foliar application of N + P express in tallest plants (226.6cm), followed by P+K (225.2 cm). Similarly to our results, combination with P and K greatly influenced the vegetative growth and plant height of maize [4]. The shortest plants (212.7 cm) were observed when, N was applied alone. The 1% nutrients at two stages each at 30 and 60 days after germination (DAG) produced taller plants (227.6 cm), while the application of 2% nutrients in one split at 30 DAG produced the dwarf plants (213.7 cm). They also found that delayed foliar spray (60 DAG) produced taller plants (203 cm), and the shortest plants (190 cm) were recorded when spray was applied at 15 DAG.

3.3 Biological yield

Nutrient combination and application time had significant effects, while interaction had no significant effects on biological yield. Among the nutrient combination, application of N+P+K produced the maximum biological yield (11333 kg ha-1), followed by N + P (10761 kg ha-1); while the lowest biological yield (9740 kg ha-1) was obtained when K was sprayed alone. Among the application times, application of nutrients in two equal splits (1% each at 30 and 60 DAG) increased the biological yield to maximum (10638 kg ha-1), while application of nutrients (2%) at 30 DAG decreased the biological yield to minimum (9895 kg ha-1). Sanjeev et al[9] reported that individual grain weight or 1000 grain weight are regarded as the basis for higher nitrogen rate can promote leaf area development during vegetative development in grains weight.

3.4 100 grain weight

Nutrient combination, application time and interaction had significant effects on the thousand grain weight in maize. Among the nutrient combinations N + P produced the maximum 100 grains weight (236.7 g), followed by N sprayed alone (231.8 g). The lowest 100 grain weight (223.3 g) was recorded when K was sprayed alone. From the application of nutrients in one split dose (2% each) at 60 DAG increased 100 grains weight (232.5 g), while application of nutrients in one split (2% each) at 30 DAG decreased 100grain weight (225.9 g) in maize. As reported that Sanjeev et al. [9] individual grain weight are basis for final economic yield from higher nitrogen rate that could promote leaf area development vegetative growth and maintaining functional leaf area.

3.5 Grain yield

Foliar application of NPK and there interaction had significant effects to produced the maximum grain yield (3287 kg/ha) followed N + P (3187 kg/ha); while the lowest grain yield (2547 kg/ha) was obtained alone K spray. Application of nutrients in two equal splits (1% each at 30 and 60 DAG) increased the grain yield to maximum (3067 kg/ha), while application of nutrients (2%) at 30 DAG decreased the grain yield to minimum (2795 kg ha). The interaction between foliar nutrients and their application time indicated that sole N application at 2% at 30 DAG increased grain yield; whereas, combined foliar application of

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N+P+K at 2% at 60 DAG or at 1% at both 30 and 60 DAG increased maize grain yield significantly. According to Girma et al. [10], maize grain yield reach to P application with increase 3000 kg/ha as control.

3.6 Harvest index (%)

Nutrient combination had significant effects with nutrient combinations; application of N + P produced the maximum harvest index (30.0%), followed by N spray alone (29.6%). Lowest harvest index (25.6%) was obtained when P was sprayed alone in maize. Such types harvest index ranged from 27.4 to 29.0% among different treatments. Foliar N-sources (urea) had significantly higher harvest index than control. Among the N source, harvest index, followed 38.4%, and control stood. Delayed application of foliar spray (60DAG) resulted in higher harvest index (38.5%), and the lowest harvest index (36.9%) was recorded when the spray was applied at 15 DAG.

The present study showed that NPK application enhanced vegetative growth of maize crop along with plant height, grain ear, grain yield and 100 grain weight comparison to control. NPK application was enhance maize crop growth and yield from stored carbohydrates which help of progressive organs under water stress condition [11]. Such type's foliar applications of P are 20 times more effective than soil application under moisture stress condition [12]. Foliar application of K under grain filling condition is increased grain yield and yield components under adverse condition of water deficit [13]. Therefore foliar application of NPK supplementation with soils are apply fertilizers but cannot replace soil fertilizers in maize growing condition [14]. Such types NPK foliar-applications are seven times more efficient when applied after sowing of maize crop in soil [12], as show a progressive effect in maize crop.

4. CONCLUSION

Maize growth and yields are more affected from foliar application of NPK under moisture stress conduction. Major NPK are improves growth, increase yield and yield components of maize under moisture stress condition, because foliar NPK application are not only provides the stability of maize under moisture stress condition but it could also provide moisture of thirsty maize plants. Such types foliar NPK are increase growth and yield along with stability of maize crop dry condition.

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EFFECT OF PESTICIDES (THIODICARB, DIMETHOATE, FOSETYL-AL AND COPPER OXYCHLORIDE) ON THE ACTIVITY OF SOIL ENZYME (DEHYDROGENASE) IN AGRICULTURAL GROUNDNUT CULTIVATED SOILS

A. Rekhapadmini[1]* and Rangaswamy. V[1]

[1]Department of Microbiology, Sri Krishnadevaraya University, Anantapuram, Andhra Pradesh

ABSTRACT: Dehydrogenases conduct a broad range of oxidative activities responsible for the degradation of organic matter and its activity is a valuable parameter for assessing the side effects of pesticides on the soil microbial biomass. In our present experimental study, the activity of dehydrogenase increased in all pesticide treated soils up to 5.0 kg ha^{-1} than the control after 21 days of incubation. Thiodicarb, dimethoate, fosetyl-Al and copper oxychloride enhanced the dehydrogenase activity significantly in 21-day incubated soil samples, whereas the enzyme activity gradually decreased with increase in period of incubation up to 35 days. Amendment of pesticides, with thiodicarb, dimethoate, fosetyl-Al and copper oxychloride in two groundnut soils resulted in inhibitory effect of dehydrogenase activity was recorded with higher concentrations 7.5 and 10 kg ha^{-1} (except with thiodicarb at 7.5 kg ha^{-1}) indicating antagonistic interactions. After 7 days of incubation, 10 - 52, 16 - 56, 02 - 59, 19 - 45% and 19 - 59, 16 - 50, 02 – 45 and 17 – 52 % increase in dehydrogenase activity was observed by the application of pesticides in black and red soils respectively, when compared to controls.

KEYWORDS: Dehydrogenases, Thiodicarb, dimethoate, fosetyl-Al and copper oxychloride.

Corresponding Author: Dr. A. Rekhapadmini***** Ph.D.
Department of Microbiology, Sri Krishnadevaraya University, Anantapuram, Andhra Pradesh
Email Address: rekha.mannepula@gmail.com

1. INTRODUCTION

Soil is a living dynamic system containing many free enzymes, immobilized extracellular enzymes and enzymes within microbial cells. There are lots of enzymes in soil environment, such as oxidoreductases, hydrolases, isomerases, lyases and ligases. Each of them plays key biochemical functions in the overall process of material and energy conversion [1]. Criteria for choosing enzyme activities as biomarker to assess soil quality is based on their sensitivity to soil management practices, importance in nutrient cycling, organic matter decomposition and bioremediation activities [2]. Pesticides are also impact soil enzymes, which are essential catalysts ruling the quality of soil life. In particular, the activity of soil enzymes control nutrient cycles and in turn, fertilization [3].

Dehydrogenase is a catalyst of aerobic respiration, and it could be used as an index for the total oxidative activities of the cell. Therefore, dehydrogenase activity in soil could be used as measurement for overall microbial activity [4, 5]. Mayanglambam [6] studied effect of the organophosphate insecticide, quinalphos, on dehydrogenase activity after 15 days of incubation in soil. Dehydrogenase activity was recovered after 90 days of treatment which may be due to adoption of soil microorganisms to counter the effect of chemical stress in hostile conditions. In our laboratory studies we recorded, the effects of pesticides (thiodicarb, dimethoate, fosetyl-Al and copper oxychloride) on the activity of soil enzyme dehydrogenase in agricultural groundnut cultivated soils.

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2. MATERIALS AND METHODS

2.1. Soils

Samples of a black clay soil and red sandy clay soils, collected from groundnut cultivated fields of Anantapur district, a semi-arid zone of Andhra Pradesh, India, to a depth of 12 cm, were air-dried and sieved through a 2-mm mesh screen before use Jackson, 1971 [7]. Physico-chemical characteristics of the two soils were analyzed using standard methods and listed in Table 1.

2.2 Pesticides

In order to determine the influence of pesticides on dehydrogenase activity, insecticides of thiodicarb, dimethoate, and fungicides of fosetyl-Al, copper oxychloride were selected in the present study on the groundnut soil.

2.3 Soil incubation

2.3.1 Dehydrogenase activity in soils

To study the effect of pesticides on dehydrogenase, 5 g of dried black clay soil and red sandy loam soil were taken separately in test tubes (12 × 125 mm) containing different concentrations of pesticides 10, 25, 50, 75, and 100 μg g^{-1} soils which are equal to 1.0, 2.5, 5.0, 7.5, and 10.0 kg ha^{-1} of field application rates. In order to maintain 60% water holding capacity (WHC), about 2 ml of deionized water was added to test tubes containing black clay soil and 1 ml into tubes containing red soil. Untreated soil samples served as controls. All the treatments, including controls were incubated in the dark at 28 ± 4°C for 1, 2, 3, 4, and 5 weeks. During the
incubation period certain amount of distilled water was added to maintain the soil WHC. Triplicate soil samples were withdrawn for the enzyme assay.

2.3.2 Assay of dehydrogenase

The method employed for the assay of dehydrogenase was developed by Casida *et al.*, 1964 [8]. This method is based on the reduction of 2, 3, 5-triphenyltetrazoliumchloride (TTC) to triphenyl formazan (TPF). Each soil sample was treated with 0.1 g of $CaCO_3$ and 1 ml of 0.18 mM aqueous solutions of TTC and incubated for 24 hours at 30°C. The TPF formed was extracted with methanol from the reaction mixture and assayed at 485 nm in a spectrophotometer.

2.4 Statistical analysis

All data were expressed on an air-dry soil basis and were averages of three replicates. The data were analyzed for significant differences ($P \leq 0.05$) between pesticide treated and untreated soils using Duncan's multiple range (DMR) test [9, 10].

3. RESULTS AND DISCUSSION

In our present experimental study, the activity of dehydrogenase increased in all pesticide treated soils up to 5.0 kg ha^{-1} than the control after 21 days of incubation (Fig. 1 and 2).

Table 1. Physico-chemical properties of soils used in the present study

Properties	Black soil	Red soil
Sand (%)	80.2	63.6
Silt (%)	13.4	23.3

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Clay (%)	6.4	13.1
pH[a]	8.0	7.5
Water holding capacity (ml g^{-1} soil)	0.45	0.33
Electrical conductivity (m.mhos)	264	228
Organic matter [b] (%)	1.85	0.054
NH_4^+- N (µg g^{-1} soil)[d]	8.42	6.69
NO_2^-- N (µg g^{-1} soil)[e]	0.56	0.41
NO_3^-- N (µg g^{-1} soil)[f]	0.92	0.81

[a]1:1.25 (Soil: water)
[b]Walkley-Black Method (Jackson, 1971)[7]
[c]Micro-Kjeldhal Method (Jackson, 1971)[7]
[d]Nesslerization method (Jackson, 1971)[7]
[e]Diazotization Method (Barnes and Folkard, 1951)[11]
[f] Brucine Method (Ranney and Bartlett, 1972)[12]

Thiodicarb, dimethoate, fosetyl-Al and copper oxychloride enhanced the dehydrogenase activity significantly in 21-day incubated soil samples, whereas the enzyme activity gradually decreased with increase in period of incubation up to 35 days (Fig. 1 and 2). Amendment of pesticides, with thiodicarb, dimethoate, fosetyl-Al and copper oxychloride in both soils resulted in inhibitory effect of dehydrogenase activity was recorded with higher concentrations 7.5 and 10 kg ha^{-1} (except with thiodicarb at 7.5 kg ha^{-1}) indicating antagonistic interactions (Tables 2 and 3). After 7 days of incubation, 10 - 52, 16 - 56, 02 - 59, 19 - 45% and 19 - 59, 16 - 50, 02 – 45 and 17 – 52 % increase in dehydrogenase activity was observed by the application of pesticides in black and red soils respectively, when compared to controls (Tables 2 and 3).

Table 2. Influence of selected insecticides thiodicarb and dimethoate and fungicides of fosetyl-Al and copper oxychloride on activity of dehydrogenase* in black soil after 7 days incubation

Pesticide concentration (kg ha^{-1})	Thiodicarb	Dimethoate	Fosety-Al	Copper oxy chloride
0.0	240a (100)	240a (100)	240a (100)	240a (100)
1.0	265b (110)	278b (116)	245a (102)	285b (119)

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2.5	315d (131)	305d (127)	280c (117)	225d (93)
5.0	366c (152)	374c (156)	382b (159)	350c (145)
7.5	304d (127)	230a (96)	200d (83)	211e (88)
10.0	205e (85)	200e (83)	170e (70)	160f (67)

* Values are µg ($NO2^-$ + $NO3^-$) – N g^{-1} soil.
Figures, in parentheses, indicate relative production percentages.
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

There are lots of enzymes in soil environment, such as oxidoreductases, hydrolases, isomerases, lyases and ligases. Each of them play key biochemical functions in the overall process of material and energy conversion [1]. Soil dehydrogenases are the major representatives of the Oxidoreductase enzymes class [1].

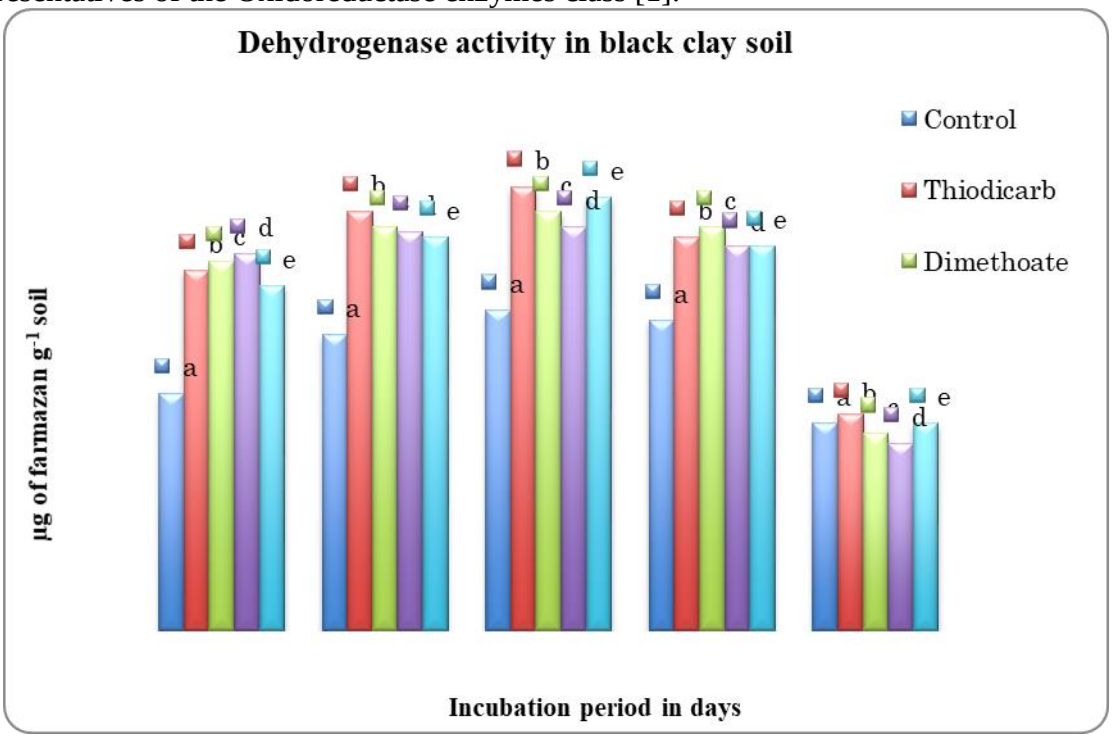

Fig.1. Influence of Thiodicarb, Dimethoate, Fosetyl-Al and Copper-oxychoride at 5.0 Kg ha^{-1} respectively on Dehydrogenase activity* in black clay soil

Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.
*Values plotted in figure are means of triplicates.

Among all enzymes in the soil environment, dehydrogenases are one of the most important and are used as an indicator of overall soil microbial activity [13,1,14], because they occur intracellular in all living microbial cells [15,16,17]. Moreover, they are tightly

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linked with microbial oxido-reduction processes [18]. Dehydrogenases do not accumulate extracellular in the soil. Dehydrogenases play a significant role in the biological oxidation of soil organic matter (OM) by transferring hydrogen from organic substrates to inorganic acceptors [19]. Many specific dehydrogenases transfer hydrogen to either nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) [20]. Throughout mentioned co-enzymes hydrogen atoms are involved in the reductive processes of biosynthesis. Due to this fact, the overall dehydrogenase activity (DHA) of a soil depends on the activities of various dehydrogenases, which are fundamental part of the enzyme system of all living microorganisms, like enzymes of the respiratory metabolism, the citrate cycle, and N metabolism [20]. Thus, DHA serves as an indicator of the microbiological redox-systems and could be considered a good and adequate measure of microbial oxidative activities in soil.

Table 3. Influence of selected insecticides thiodicarb and dimethoate and fungicides of fosetyl-Al and copper oxychloride on activity of dehydrogenase* in red soil after 7 days incubation

Pesticide concentration (kg ha^{-1})	Thiodicarb	Dimethoate	Fosety-Al	Copper oxy chloride
0.0	210a (100)	210a (100)	210a (100)	210a (100)
1.0	250b (119)	245b (116)	214a (102)	245b (117)
2.5	210a (100)	220a (105)	247c (118)	278c (152)
5.0	335c (159)	315c (150)	305b (145)	320d (132)
7.5	170d (81)	200a (95)	142d (68)	240b (114)
10.0	120e (57)	155d (74)	96e (46)	160e (76)

* Values are µg ($NO2^-$ + $NO3^-$) – N g^{-1} soil.
Figures, in parentheses, indicate relative production percentages.
Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

Srinivasulu and Rangaswamy (2012) [21] reported that the activity of dehydrogenase increased in all pesticide treated soils up to 2.5 kg ha^{-1} than the control after 21 days of incubation. Monocrotophos either singly or in combination with mancozeb (fungicide) improved the dehydrogenase activity significantly in 21-day incubated soil samples, whereas the enzyme activity gradually decreased with increase in period of incubation up to 35 days. Likewise, individual application of metalaxyl (fungicide) increased the dehydrogenase activity initially in fungicide-treated (40–80 µg g^{-1}) soil and then gradually decreased after 30 days [22]. In contrast, Mayanglambam *et al*., (2005) [6] observed 30 % inhibition in dehydrogenase activity in quinalphos treated soil after 15 days of incubation. Monocrotophos

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and chlorpyrifos at concentrations ranging from 1.0 to 2.5 kg ha^{-1} increased the dehydrogenase activity gradually and reached maximum at the concentration of 2.5 kg ha^{-1} in both vertisol and laterite soils. Amendment of both soils with monocrotophos and chlorpyrifos above 2.5 kg ha^{-1} resulted in minimum dehydrogenase activity while higher concentrations (7.5 and 10 kg ha^{-1}) showed inhibitory effect indicating antagonistic interaction. Similarly, Nweke *et al.*, (2007) [23] also reported that atrazine and northrin (herbicides) stimulated the dehydrogenase activity at lower dose (0.2 %) and inhibited it at higher concentration (0.55 %) in rhizoplane microbial community. After 10 days of incubation, 10 to 52 and 19 to 59, 2 to 59 and 2 to 45 % increase in dehydrogenase activity was observed by the application of pesticides in vertisol and laterite soils respectively, when compared with controls Likewise, Singh and Kumar (2008) [24] observed that acetamiprid increased dehydrogenase activity up to 22 % after first insecticide application.

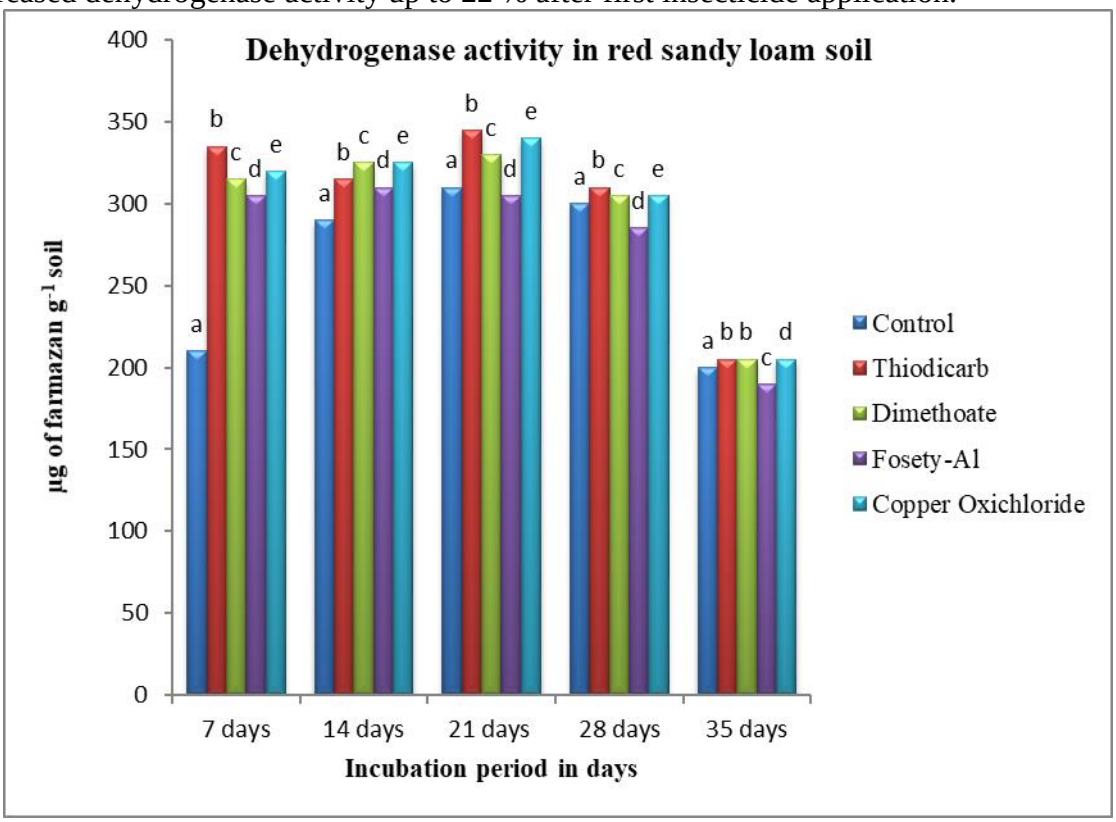

Fig 2. Influence of Thiodicarb, Dimethoate, Fosetyl-Al and Copper-oxychoride at 5.0 Kg ha^{-1}respectively on Dehydrogenase activity* in red sandy loam soil

Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$) from each other according to Duncan's multiple range (DMR) test.

*Values plotted in figure are means of triplicates.

4. CONCLUSION

Pesticides are also vital to sustainable development in many countries in the developing world, by protecting crops such as groundnut and preventing financial ruin by infestations of pests. The use of pesticides has increased many folds over the past few decades. According to an estimate, about 5.2 billion pounds of pesticides are used worldwide per year [25]. Pesticides are widely used against a range of pests infesting agricultural crops. Globally, about 3×10^9 kg of pesticides is applied annually with a purchase price of nearly $40 billion each year [26]. The amount of applied pesticides reaching the target organism is about 0.1%

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while the remaining bulk contaminates the soil environment [27]. With the growing use of pesticides in contemporary agriculture, the issue of the impact of these chemicals on the composition of soil microorganisms and the processes they direct has received more attention [28]. The applied pesticides may harm the indigenous microorganisms, disturb soil ecosystem and thus, may affect human health by entering in the food chain. Adverse impacts of pesticides on soil microbial diversity and activities have been described by many researchers [29, 30, 31and 32]. Similarly, pesticides also influences soil biochemical processes driven by microbial and enzymatic reactions. The microbial mineralization of organic compounds and associated bio transformations such as nutrient dynamics and their bioavailability are also more or less adversely affected by the pesticides [33]. The applied pesticides also reduce soil enzymatic activities that act as a ‘‘biological index’’ of soil fertility and biological processes in the soil environment [34].

However, no literature was found regarding the interaction of present selected pesticides viz., thiodicarb, dimethoate, fosetyl and copper oxychloride on enzymatic activities of two different soils. Therefore, the impact of these pesticides on soil enzyme like dehydrogenase, in two groundnut soils was determined. The dehydrogenase enzyme activity was measured in both soil samples at 7-day interval by triphenyltetrazolium chloride (TTC) reduction method under the influence of selected pesticides. The rate of dehydrogenase enzyme activity were further analysed after 14, 21, 28, and 35 days of incubation, with the stimulatory concentration (2.5 or 5.0 kg ha^{-1}) of selected pesticides.

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PRODUCTION AND SENSORY EVALUATION OF TROPICAL FRUIT WINE FROM *FICUS RACEMOSA* AND *GARCINIA INDICA*

Venkata Krishna Bayineni[1]*, Pethannan Rajarajan[1], P Malaiyarasa Pandian[1]
[1] Department of Microbiology, Center for Research & PG Studies, Indian Academy Degree College, Hennur Cross, Kalyan Nagar, Bangalore-560043, India.

ABSTRACT: Fruit wine making is one of the most commercially prosperous biotechnological processes which has got global appreciation. *Garcinia indica*fruit is known for its high content of hydroxycitric acid and anthocyanins and *Ficus racemosa* fruit holds an excellent source of phenolic compounds. Thus, the aim of this study was to produce exotic tropical fruit wine from *F. racemosa*by blending with *G. indica*. Mixed wine of these fruits was produced by ten different must blends fermented with *Saccharomyces cerevisiae*. During fermentation aliquot sample for the analysis of pH, temperature, alcohol content and reducing sugars, were carried out using standard procedures. The pH of the fruit must during the fermentation period ranged from 3.3 to 4.1 and consistent increase in alcohol content was observed with time. At the end of 22nd day of fermentation, the concentration of alcohol was observed to be 4.4%. Here we also report the sensory evaluation of the wines obtained from ten combinations of these fruit blends. Overall results revealed that best wine was obtained with 1:2 blend of the two fruits (*F. racemosa* and *G. indica*). Thus, mixed wine produced from the underutilized fruits of *F. racemosa* and *G. indica* using *S. cerevisiae*, was shown to be a possible potential candidate for coloured exotic fruit wine production.

KEY WORDS: Fruit wine: *Ficus racemosa: Garcinia indica*: *Saccharomyces cerevisiae*: Sensory evaluation

***Corresponding author:** Venkata Krishna Bayineni**,**
Tel No. +919035215077
Email address: krishnabayineni@gmail.com

1. INTRODUCTION

Fermentation is biotechnology in which desirable microorganisms are used in the production of value-added products with health benefits and is one of the oldest forms of food preservation technology in the world. Fermentation is a potent tool in the development of new products with modified chemical and physical properties, improved sensory qualities and nutrition besides therapeutic value. India is one of the largest producers of fruits in the world. Major category fruits are extensively cultivated in most tropical and subtropical countries. Minor category fruits are not so extensively cultivated for consumption and trade tends to be more limited. Considerable research efforts have been made towards fermented wines from fruits and vegetables [1-2]. Fermented foods are more nutritious than unfermented ones due to synthesis or release of nutrients by some of the microorganisms employed [3]. There are several research reports on wine preparation from different fruits like apple, pomegranate, plum, apricot, strawberry, litchi, amla, guava, jamun, sapota, carambola, orange *etc*. [4-7]. Several underutilized fruits are grown which are known for their therapeutic and nutritive values with an excellent flavor and attractive color for lower prices and have a great potential for processing. The most important underutilized fruits are aonla, bael, fig fruit, jackfruit, jamun, kokum, karondaphalsa *etc*. are having considerable economic importance [8].
Garcinia indica (Kokum fruit) belongs to the family Cruciaceae and genus *Garcinia* [9] is indigenous to the Western Ghats region of southern India and is distributed in Maharastra, Goa, Gujarat west Bengal, Assam and Karnataka with major area in Maharashtra [10]. Kokum is a seasonal fruit and the yields are very high during summer. The fruit is used in its

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dried form to develop many value added products. The pulp and the rind of the fruit can be used in several ways. The dried rind contains 2–3 per cent anthocyanin pigments which are promising source of natural colourant for acid foods. Kokum fruit rind is rich source of hydroxyl citric acid which is unique, potent, metabolic regulator of obesity and helpful in treating many cardiovascular risk factors associated with abdominal obesity [11]. It also finds place in ayurveda as therapeutic agent and its effect includes burns, piles, allergies, sunstroke, diarrhea, dysentery, tumor and cardiac diseases, *etc*. Kokum is a rich source of beneficial compounds like HCA (hydroxyl citric acid), garcinol, citric acid, malic acid, anthocyanin pigments and ascorbic acid [12]. The proximate composition of kokum rind contains moisture 30% protein 1.92% crude fiber 14.28%, pectin 5.71 %, crude fat 10.0%, hydroxyl citric acid 22.80% and carbohydrate 36.40% [13]. Kokum juice fermented by yeast (*Saccharomyces ellipsoideus* No 101) is very good therapeutic agent and good antioxidant activity [14].

Ficus racemosa (Fig fruit) belongs to family Moraceae, which is native to Australia, South-East Asia and the Indian subcontinent and frequently found around the water streams [15-16]. Fig fruits are pyriform or subglobose, red when ripe, borne in large clusters on branches [17]. All parts of the *F. racemosa* plants possess have potent anti-diabetic properties [18]. There is high potential for the development of fermented product of fig fruits which contains symbiotic affects from kokum fruits with health benefits.

Traditionally, the fermentation is carried out by *S. cereviceae* for the production of wine. The quality of wine depends upon a number of factors like cultivars, adequate sugar level, acid content, color, and aroma and strains used [19]. Lactic acid fermented beverage from kokum juice using the REFERENCES strain *Lactobacillus acidophilus* has been developed [20]. However, wine production from Kokum and fig fruits have not been explored very much owing to their low sugar content; hence it is required to add an external source of sugar to facilitate the fermentative process by yeast. For this purpose the raw materials rich in sugars is blended to improve the quality of flavor, chemical characters and nutrient content for producing tropical fruit wines with a natural aroma and a desirable alcoholic content.

The present work reports on kokum–fig fruit blends for wine production using *S. cerevisiae* NCIM 3215 and the chemical properties including sensory evaluation of the various wines obtained from ten combinations of kokum fig-kokum blends. Overall results revealed that kokum and fig fruit has a good potential for winemaking and in addition to its unique red-pink colour and anti-obesity compound makes this wine a healthy option for consumers.

2. MATERIALS AND METHODS

2.1. Microorganism and inoculum preparation

The yeast culture (*Saccharomyces cerevisiae* NCIM 3215) was obtained from NCIM (National
Collection of Industrial Microorganism) Pune, India. It was maintained on yeast malt extract agar (YMEA, HiMedia Laboratories, Mumbai, India) and sub-cultured after every three months. The inoculum was prepared by growing culture in a 250 mL flask containing 100 mL media (malt extract 3 g/L, glucose 10 g/L, yeast extract 3 g/L and peptone 5 g/L) and then incubated at 25 °C for 48 h.

2.2. Preparation of “mixed must” and fermentation

The ripened fruits of kokum and fig were washed two times with distilled water to remove dirt and other impurities. Then the fruits were weighed, washed, manually deseeded, rewashed, and then blended using a sterile blender, and then filtered. The necessary amount of water and 25 % granulated sugar was added for juice preparation. Different blends were

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prepared by mixing kokum and fig juice filtrates in different proportions (Table 1) and the juice blends prepared were coded as sample 1 – 10. Potassium metabisulfite (50 mg/L) was added to all juice blends and the juice was held for 24 h at 4 °C to avoid the growth of unwanted microbes. Musts were inoculated with 5% v/v *S. cerevisiae* from the prepared inoculum. Fermentation was carried out at 28 °C and pH 4.5 until constant Brix readings were observed. Aliquots of the fermented juice obtained at different time intervals and used for pH, reducing sugar, alcohol and sensory analysis.

Table 1. Sample codes for different blends of fig and kokum juice combinations used

Sample code	Proportion of fruits used (Fig : Kokum)
Sample 1	3:1
Sample 2	2.5:1
Sample 3	2 : 1
Sample 4	1.5 : 1
Sample 5	1 : 1
Sample 6	1 : 1.5
Sample 7	1 : 2
Sample 8	1 : 2.5
Sample 9	1 : 3
Sample 10	1 : 3.5

2.3. Downstream processing

Wine samples were centrifuged at 5000 rpm for 15 min at 20°C and then filtered through a 0.4 μm filter and bottled in amber-colored bottles with screw caps.

2.4. Physiochemical parameters

The alcohol content of the wine samples was measured using alcoholmeter (Leimco, Mumbai, India). The pH measurements were taken using a portable digital pH meter by taking 10 ml of the "must" into a sterile beaker.The quantitative estimation of reducing sugar of the wine samples was determined by of 3, 5-Dinitrosalicyclic acid (DNS) method described by Miller (1959) [21].

2.5. Sensory evaluation

The sensory analysis of different blends was conducted to evaluate the aroma and acceptance by selected panel of members through organoleptic procedures after aging for 22 days. Observations were recorded for appearance, color, aroma, clarity, taste and general quality on a 5 point scale with 5 points for excellent quality and 1 point for bad quality. Wines were served at 20 °C in wine glasses.

3. RESULTS

3.1. Physiochemical properties

The *pH* of all wine samples was within the acidic range throughout the period of fermentation irrespective of the proportion of fruit blends used. *pH* ranged from 4.11 to 3.32 (Table 2). As shown in table 2, a steady increase in alcohol content was observed in all the fruit blends throughout the period of fermentation. At the end of the 22nd day, of the fermentation, the concentration of alcohol in the fruit wines was observed to be around 4.4 %. In the case of

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reducing sugar of the wine samples during the period of fermentation, the values observed to be decreasing. It was high on the first day which was the start of the wine fermentation and due to the presence of sugar in the fermenting wine, the reduction in the reducing sugar is due to the activities of the fermenting yeast on the wine and the production of alcohol. The reducing sugar ranged from 0.36 g/ml on the first day to 0.072 g/ml on the 22nd day.

Table 2. Physicochemical properties of wine samples

Sample	pH				Reducing sugars (g/ml)				Alcohol			
	5th day	10th day	17th day	22nd day	5th day	10th day	17th day	22nd day	5th day	10th day	17th day	22nd day
1	4.26±0.015	3.89±0.013	3.51±0.011	3.35±0.012	0.23±0.013	0.12±0.011	0.09±0.011	0.076±0.013	2.31±0.011	3.56±0.013	3.85±0.010	3.98±0.012
2	4.29±0.011	3.95±0.011	3.62±0.012	3.40±0.011	0.25±0.011	0.15±0.013	0.10±0.012	0.079±0.013	2.25±0.013	3.74±0.014	4.02±0.011	4.10±0.013
3	4.38±0.013	3.89±0.012	3.56±0.014	3.32±0.013	0.27±0.014	0.21±0.011	0.12±0.013	0.078±0.014	2.39±0.014	3.63±0.010	3.85± 0.013	3.91±0.011
4	4.25±0.012	4.06±0.014	3.68±0.011	3.36±0.011	0.28±0.013	0.17±0.011	0.12±0.014	0.080±0.013	2.43±0.011	3.82±0.011	4.15± 0.014	4.36±0.013
5	4.32±0.012	3.98±0.011	3.71±0.013	3.32±0.014	0.31±0.015	0.16±0.014	0.11±0.010	0.076±0.011	2.53±0.013	3.51±0.014	4.02± 0.013	4.21±0.011
6	4.35±0.012	4.02±0.011	3.51±0.011	3.39±0.015	0.32±0.011	0.19±0.013	0.13±0.011	0.086±0.013	2.36±0.014	3.96±0.012	4.12± 0.010	4.35±0.010
7	4.19±0.011	4.06±0.013	3.65±0.014	3.23±0.013	0.34±0.014	0.14±0.011	0.10±0.010	0.085±0.014	2.43±0.013	3.62±0.011	4.06± 0.012	4.41±0.011
8	4.35±0.010	4.10±0.012	3.51±0.011	3.28±0.011	0.35±0.013	0.16±0.011	0.11±0.011	0.089±0.013	2.21±0.011	3.55±0.010	3.86± 0.012	4.29±0.013
9	4.22±0.011	3.99±0.013	3.64±0.012	3.35±0.013	0.35±0.011	0.12±0.010	0.13±0.011	0.087±0.014	2.45±0.010	3.88±0.011	4.05± 0.011	4.38±0.010
10	4.09±0.012	4.08±0.011	3.48±0.013	3.38±0.014	0.36±0.013	0.21±0.013	0.14±0.010	0.091±0.012	2.31±0.011	3.49±0.013	3.94± 0.010	4.21±0.011

Values represent mean ± Standard deviation (SD)

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3.2. Sensory analysis

The mean scores of organoleptic evaluation for each category of the 6 parameters for all 10 wine samples are given in table 3. The results showed that in all the wines under comparison, the wine produced from 1:2 proportion of fig and kokum fruits secured highest score followed by wine produced from 1:2.5 blend. The lowest scores were obtained for sample 1, 2 and 10 (Table 3). The acceptance of fig–kokum blended wine suggesting its market potential. Sensory analysis revealed that the wine prepared from the must (1:2 proportion of fig and kokum) had acceptable appearance, color, aroma, clarity, taste and general quality (score, 24.54 of 30 points).

Table 3. Sensory evaluation score of the fig-kokum wines produced from different must blends

Wine sample codes	Parameter						Total score (30)
	Appearance	Color	Aroma	Clarity	Taste	General quality	
Sample 1	3.24±0.021	3.25 ± 0.062	3.24 ± 0.032	3.02 ± 0.052	3.21±0.092	2.81 ± 0.062	18.78±0.052
Sample 2	3.39±0.025	3.41 ± 0.032	3.32 ± 0.042	2.92 ± 0.032	3.28±0.052	2.98 ± 0.033	19.33±0.036
Sample 3	4.02±0.027	3.84± 0.034	3.52± 0.037	3.13± 0.022	3.82±0.032	3.35± 0.038	21.68±0.031
Sample 4	3.96±0.019	3.95± 0.027	3.65± 0.021	3.42± 0.028	3.62±0.046	3.16± 0.036	21.76±0.024
Sample 5	3.56±0.025	4.01± 0.029	3.46± 0.035	3.51± 0.020	4.01±0.047	3.52± 0.046	22.07±0.028
Sample 6	3.84±0.017	3.68± 0.029	3.95± 0.024	3.36± 0.021	3.93±0.023	3.22± 0.048	21.98±0.021
Sample7	4.13±0.023	4.35± 0.021	3.92± 0.029	3.66± 0.034	4.12±0.026	4.36± 0.052	24.54±0.025
Sample 8	3.76±0.025	4.21± 0.021	3.73± 0.036	3.36± 0.036	3.58±0.034	4.04± 0.053	22.68±0.029
Sample 9	3.65±0.019	4.13± 0.033	3.64± 0.034	3.05± 0.038	3.56±0.036	3.81± 0.042	21.84±0.031
Sample 10	2.85±0.020	3.83± 0.039	3.41± 0.051	3.21± 0.042	3.21±0.045	3.36± 0.047	19.87±0.035

Values represent mean ± Standard deviation (SD)

4. DISCUSSION

In this research work, the choice of the fruits: kokum and fig fruits were deliberate. This work has shown that the *pH* ranges of the mixed fruit juice blends used for the production of the wine were 4.11 – 3.32, while there was no significant difference in the reducing sugars amongst the ten samples. Studies have shown that during fermentation of fruits, low pH will inhibit the growth of spoilage microbes and is known to give competitive advantage in natural environments for the growth of desirable microorganisms.

The major problem associated with the use of tropical fruits for wine production is their low sugar content [22]. In order to supplement the sugar content of the “musts” granulated sugar was added as additives. Remarkable amount of alcohol was produced during the process of fermentation and this trend was consistent during the course. In general, the percentage alcohol in the different fermented fruit wines by the yeast strain was above 2% which is comparable with moderate grape wines [23-24]. The performance and potential of the yeast strain was measured by the amount of alcohol produced which are known to impart pleasant aromas by producing important precursors for the formation of esters [25]. Reports have shown that some of the by-products formed in addition to ethanol during alcoholic fermentation include carbonyl compounds, alcohols, esters, acids and acetals will influence the quality of the finished product [26].In our study, it was observed that the alcoholic content increased from the initial day to the end of the fermentation due to production of ethanol during the process of fermentation. These results agree with the reports of [27-29].

In this study, pH of the fruit wine throughout the period of fermentation ranged from 3.32 to 4.11. A similar observation has been reported by Reena *et al.* (2016) [30]; in their study on kokum fruit, the acidity in kokum contributed to lower pH values. The reported type and aroma produced during wine production depends on yeast, environmental factors and

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physico-chemical characteristics of the "musts".

5. CONCLUSION

The research work highlights the important fermentation capabilities of neglected and underutilized fruits of *Ficus racemosa* and *Garcinia indica* in their combinations. These underutilized fruit trees can be fully explored to the grass root level. However, elaborate research should be conducted to reveal the other nutritional parameters (protein, carbohydrates, fats, fibers, minerals and other vitamins) of the fermented wine for the effective utilization. The fermentation parameters should be conducted which reduces the production cost and increases the quality of product.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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COMPUTATION OF GENETIC CORRELATION BETWEEN DIETARY SUPPLEMENTS WITH LIFE HISTORY AND METRICTRAITS IN PRE AND POST-ADULT STAGES OF *DROSOPHILA*

Neethu B.K[1], Harini B.P[2]
[1]Department of life science, Garden City University,Bengaluru-560049
[2]Department of Zoology,Bangalore University,Bengaluru-560056

ABSTRACT: Morphological, physiological triats such as behaviour, metric have shown less heredity than life history traits such as fecundity and longevity in females than males. Statistical correlation was done to analyse the consequences of vareint diets on genetic and morph phenotypic variations in the morphological and life-history traits of *Drosophila* species in parent and F1 generation.There were significant positive and negative (r>0.01 and 0.05) genetic correlations between dietary concentrations and life history traits.In males the the dietary concentraions showed positive correlation with morpho phenotypic and genetic variations and in female the results showed contradictive correlation, When supplemented with varied concentration of diets. The parental effects has upheld the successive generation for the better fitness traits on supplementation with varied diets than raised in control diet. The results reveals that, *D. n. nasuta* and *D. s. sulfurigaster*on exposure to varied nutritional supplementation has shown significant effects on morpho phenotypic and genetic variations to the diverse diet concentrations in pre-adult and post-adult stages of *Drosophila*. Which indicates that trade-off between fitness traits may exist when dietary content is varied.

KEYWORDS: Genetic Correlation; Life history trait ; Metric traits; Diet.

Coressponding author: Dr.B.PHarini,Associate Professor
Bangalore University,Bangalore Karnataka India
Email id:dr.bpharini@gmail.com

1.INTRODUCTION

Animals obtain energy and nutrients from food, so diet can be considered a key factor that potentially affects all life-history components [1]. Experimental modifications of animal diets have played a key role in the study of how organisms adjust their energy allocation [2]. The amount and quality of nutrients intake by organisms have a strong impact on life-history traits, such as disease vulnerability, fertility, reproduction, longevity and stress resistance [3].In insects, restricted access to dietary protein during the growth and development phase of the life cycle typically results in reduced body size [4], but effects on viability vary across taxa and studies, apparently reflecting nonlinear responses to protein concentration [5]. Nonetheless, there is some evidence that, as in the adult diet, dietary protein during development mediates trade-offs between fitness components.

Dietary restriction (DR) is intake of one or more components (typically macronutrients) with minimal to no reduction in total caloric intake – is another alternative to caloric restriction (CR). While research suggests that neither carbohydrate restriction nor lipid restriction extend life ,protein restriction increases maximum lifespan by roughly 20% [6]. This extension of life may be solely due to the reduction of the amino acid methionine [7]. Dietary restriction (DR) refers to a moderate reduction of food intake that leads to extension of life span beyond that of normal, healthy individuals.

Dietary restriction is implemented by dilution of the diet, without compensation of food intake rates [8], resulting in increased lifespan and reduced fecundity, measured as egg laying . In nature, *Drosophila* eat yeasts and, although many manipulations of dietary balance can

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alter lifespan [3], enhanced longevity by dietary restriction is modulated almost exclusively by dietary yeast, independent of calorie intake [9] Diet restricted animals live longer either because eating less extends life, or because the normal nutritional regime in the laboratory is harmfully nutrient- rich in relation to requirements [10].

Nutrition plays prominent roles in aging, health, metabolism, and disease [11]. Despite the key advantages of *Drosophila* as a model organism, its food consumption remains challenging to measure. As a result, this crucial parameter is often ignored or poorly characterized using rough feeding estimates and qualitative observations as surrogates for quantitative food intake assays, leaving doubt as to the significance of some studies [12]. Behaviours such as eating, mating, and sleeping that are observed in humans are also seen in *Drosophila*. Therefore, the possible effect of genetics upon human behaviour can also be assessed. Insects such as the fruit fly, *Drosophila melanogaster*, rely on contact chemosensation to detect nutrient-rich foods, to avoid consuming toxic chemicals, and to select mates and hospitable zones to deposit eggs.

Flies sense tastants and non-volatile pheromones through gustatory bristles and pegs distributed on multiple body parts including the proboscis, wing margins, legs, and ovipositor. In *Drosophila*, the taste neurons located in sensilla in several body regions sense and distinguish nutritive substances such as sugars, amino acids, and low salt, and potentially harmful ones such as high salt, acids,sugar and a diverse variety of bitter compounds. The gustatory system allows animals to detect chemical compounds in the environment and determine their value as potential food sources. To make this assessment, animals detect two different features of taste stimuli with the gustatory system: the concentration and the quality of a taste compound. In humans, taste concentration is perceived as intensity and taste quality as a component of flavor. Determining how these two features of taste stimuli are encoded by the nervous system and used to direct behavior is central for the neural basis of taste perception[13].

Genetic analysis of variation among individuals within a population in nature may, on the other hand, show the susceptibility of the character to evolution by natural selection and its sensitivity to environmental variation not seen in the laboratory. Measuring heritabilities and genetic correlations in nature also allows estimation of the rates and directions of shortterm evolution [14], reconstruction of historical patterns of natural selection [15]. The relative contributions of genes and environment to phenotypic variation of *Drosophila* in the laboratory have been widely studied, but little is known of their role in free-living populations. Estimation of heritability in nature requires rearing of family groups in the wild, a procedure not feasible with *Drosophila*. Such heritabilities might be approximated by correlating phenotypic characters of wildcaught parents with those of their laboratory-reared offspring, a method employed [16] in a study of wing length in *Drosophila melanogaster*.

In addition, it is important to know whether heritable variation is as abundant in nature as in domesticated and laboratory populations [17], perhaps environmental variation in the wild results in much lower heritability. Such “natural heritability” studies can be done by correlating either parental and offspring characters in nature [18] (cross-fostering is necessary to eliminate any effects of a common family environment) or correlating the characters of wild-caught individuals with those of their laboratory-reared offspring [19]. Estimates of “natural heritability” are at present limited to vertebrates.

Parental genotype and environment often influence offspring fitness through non-genetically transmitted parental effects. Such effects may be maladaptive, e.g. malnourished parents may produce offspring of poorer quality (parental stress hypothesis). However, parents may also respond to environmental cues in ways that enhance offspring fitness. In particular, if the nutritional conditions experienced by the mother and offspring are positively correlated, mothers subject to nutritional stress would be favoured to induce plastic changes in the

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offspring that make the latter more tolerant to nutritional stress. This adaptive hypothesis thus predicts that fitness of offspring on poor diet would be enhanced if their parents also experienced poor diet [20].

The most obvious way by which environmental variation may influence body condition and fecundity is via nutritional effects resulting from variability in food type availability. In general terms, diet effect can be classified as either quantitative (i.e. food availability) or qualitative (i.e. food composition). The quantitative effects are evident since animals obtain energy and other nutritional requirements from food. Thus, under a natural range of conditions there is a positive correlation between food availability and body condition or fecundity [21].

Most animal and plant species are complexes of subdivided, local populations which differ from each other genetically to some extent. These populations are sometimes named race or subspecies according to the extent of differences between populations. In these cases, groups of populations are usually allopatric, and geographic isolation keeps the gene exchange infrequent. By contrast, species are able to maintain their genetic integrity despite of sympatric co-existence. put forward the `geographic speciation' model: geographic isolation of two populations is a necessary prerequisite in time to the development of reproductive isolating mechanisms[22].

The *Drosophila nasuta*subgroup, belonging to the *Drosophila immigrans* species group, includes more than ten species which are morphologically similar, distributed in Pacific-Australasian and the pan-Indian Ocean areas. A number of these populations in different continental areas and islands of the Pacific were demonstrated to have diverged to the point of being separate sibling species [23]. One of the species, *Drosophila sulfurigaster* has the largest distribution among the *D.* nasutasubgroup, and consists of four subspecies. These subspecies are found allopatrically according to the definition of subspecies by [24].

Studies concerned with the impact of nutrition often assesed the physiological and morphological responses of individuals exposed to different quality and amount of nutrients. With this above citations the present work has been undertaken to study the dietary restriction in the form of variable nutritional composition supplemented to two species of genus *Drosophila* namely *D. n. nasuta*and *D. s. sulfurigaster*which are evolved allopatrically, thereby to record whether the specificity of nutritional intake has any impact on life history traits traits viz. Fecundity and longevity and behavioural traits as gustatory and metric triats for thiergenetic levels in pre and post adult stages in correlation to dietary supplements.

2. MATERIALS AND METHODS

2.1 *Drosophila* culture

Drosophila nasutanasuta and *Drosophila sulfurigastersulfurigaster* (F0 and F1 generation) stocks were maintained in an uncrowded culture condition at 22±1˚C, 70% humidity and 12h: 12h light and dark cycles in standard wheat cream agar medium. From the parental stock the virgin females and unmated males were collected within six hours of eclosion and were aged for two days. Then they were allow to mate and these mated females and males were taken to perform the experiments.

2.2 Experimental diets

The dietary media were based on a cornmeal-sugar- medium containing a base of 0.5% (w/v) agar, 1% (w/ v) cornmeal, 0.75% (v/v) propionic acid, with single macronutrient i.e., concentrations of glucose (1%, 2%, and 3%, all g/L), Min et al., 2006, brewer’s yeast (5%,15% and 25%, all g/L), [25] and methionine (0.01%, 0.02%,0.03%g/L) [26] along with Dietary restricted(DR) diets i.e.30g of glucose with varied concentration of brewer’s yeast

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[27] and 30g of glucose with varied concentration methionine[26] along with control. Food was dispensed into vials (2 mL) and stored at 24±1 °C.

2.3 Gustatory feeding assay

The method described [28] was adopted for gustatory assay. Larvae and adult flies were reared in media supplemented with experimental diet and control media. To perform the feeding assay, the larvae and flies were starved for 2 hours, each experimental group were transferred into the vials containing the specific diets with bromophenol blue dye (0.05% wt/vol) and were fed for 10 minutes. The fed flies were etherized, washed with phosphate-buffer saline (PBS), and homogenized with 1 ml of distilled water. The absorbance measured at 595nm using a spectrophotometer.

2.4. Fecundity assay

Fecundity was recorded by counting number of eggs laid. Flies were successively transferred into fresh vials containing media (Experimental diets and control) every alternate day for 6 days. The same sets of vials were assessed at each interval for the number of eggs laid to record for fecundity. Further the pupal viability was evidenced for pupation. Followed by hatchability, to account for the eclosion of adult flies from pupa to trace for the fertility and total productivity [29].

2.5 Lifespan assay

Synchronous cultures of 2–3-day-old files were obtained as described earlier and transferred into vials containing experimental and control diets. Each group including the control had 10 replicates with 14–20 flies per vial with equal sex ratio. Flies were transferred to fresh media every alternate day. Dead flies were counted and removed daily throughout the experiment [30] and accorded for longevity.

2.6 Morphometric measurements

Wing length is an index of body size, accordingly the left wings of thirty individual males and females were mounted on slides with a drop of 70% glycerol and measured from the proximal to distal end along the length of costa and subcostal longitudinal vein [31] under motic stereo microscope fitted with a 3.0MP digital camera Motic Images plus 2.0 ML is multi-media software offered by Motic Ltd.

2.7 Statistical analysis

Log-rank tests of survival data and correlation analyses of non-linear relationship for genetic correlation between dietary supplements with feeding behavior, metric traits and life history traits in pre-adult and post-adult stages.was also tested using Spearman's correlation coefficient). Scatter plots were fitted using Excel (Microsoft) using linear trendline.

3. RESULTS

There were significant positive and negative genetic correlations between dietary concentrations and life history traits. When supplemented with varied concentration of diets i.e. single concentration of glucose (i.e. 1%, 2%, 3% g/L), the male flies showed negative correlation between gustatory feeding r=-582 and lifespan r=-832 whereas supplemented diet did not show any correlation to body size, while gustatory feeding showed positive correlation to body size and lifespan significant at r=864,844 ($P<0.01$) in both *D. n. nasuta*and *D.s.sulfurigaster*species (F0 and F1 generation) **Figure.1-4**.

On supplementation of only brewer's yeast (i.e. 5%, 15%, 25% g/L) the male flies negatively correlated to gustatory feeding r=-947, while positive correlation for lifespan r=454 and body size r=388 in *D. n. nasuta*and *D.s.sulfurigaster*species (F0 and F1 generation) **Figure.5-8.**

When male flies were fed with single concentration of methionine diet (i.e.0.01%,0.02%,0.03% g/L) showed positive correlation between gustatory feeding, lifespan with body size significant at $P<0.01$ in *D. n. nasuta*(F0 and F1 generation) whereas,

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*D.s.sulfurigaster*species (F0 and F1 generation) supplemented concentrations showed positive correlation to lifespan and negative correlation to gustatory feeding with no correlation to body size, while gustatory feeding showed positive correlation to body size and negatively correlated to lifespan significant at P<0.01 **Figure.9-12**

When males were supplemented DR diets including 30g/L glucose with Yeast and 30g/L glucose with methionine in both *D. n. nasuta*and *D. s. sulfurigaster*species (F0 and F1 generation), showed positive correlation to lifespan and negatively correlated to gustatory feeding, showing insignificant to body size as shown in **Figure.13-20**.

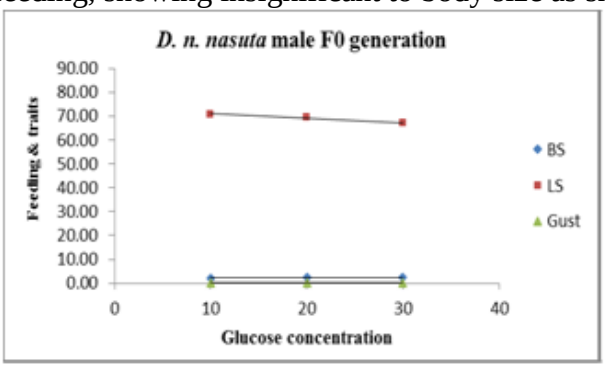

Figure 1

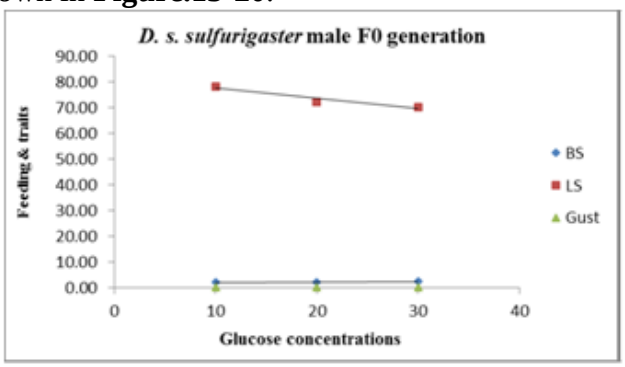

Figure 2

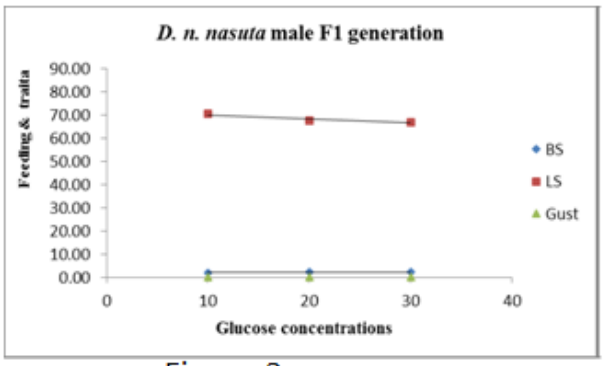

Figure 3

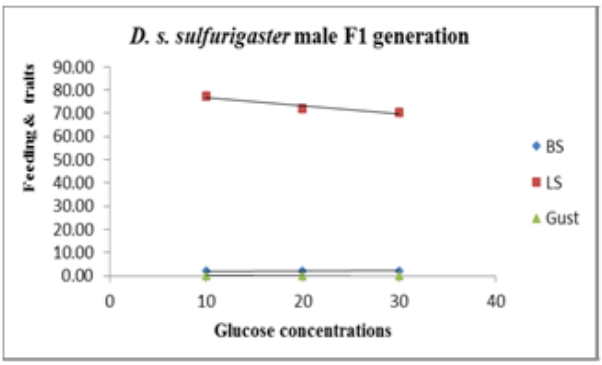

Figure 4

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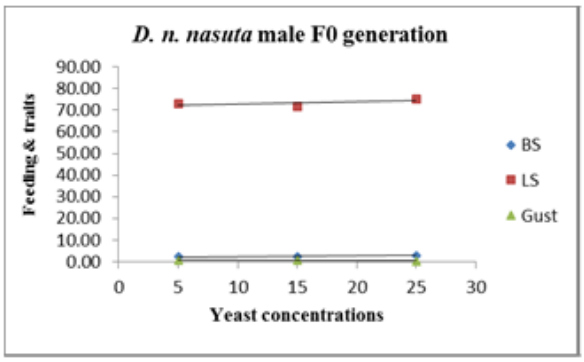

Figure 5

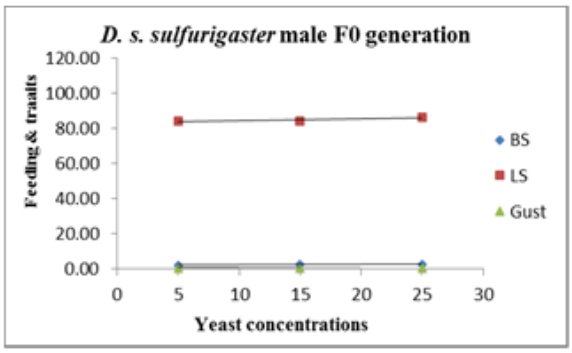

Figure 6

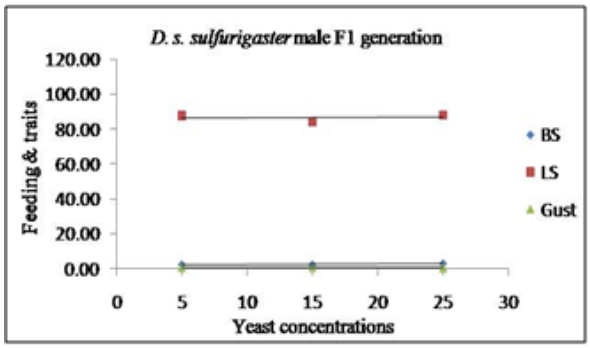

Figure 7

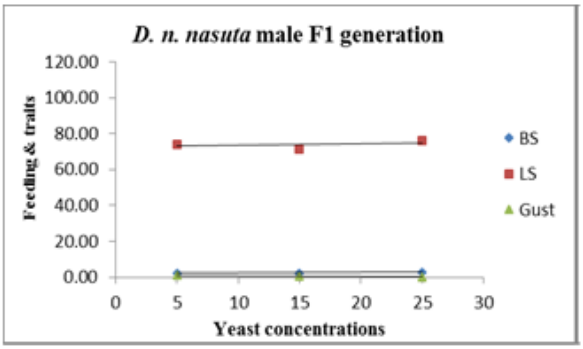

Figure 8

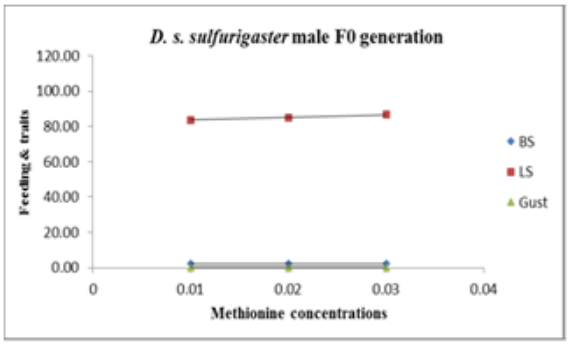

Figure 9

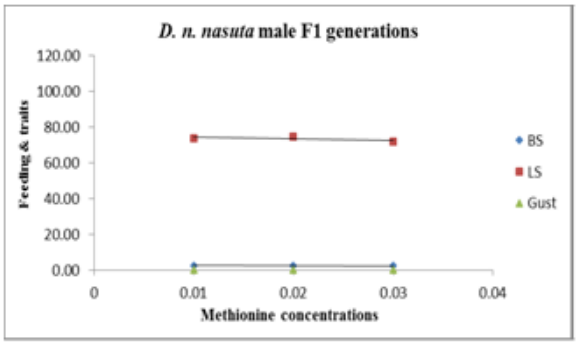

Figure 10

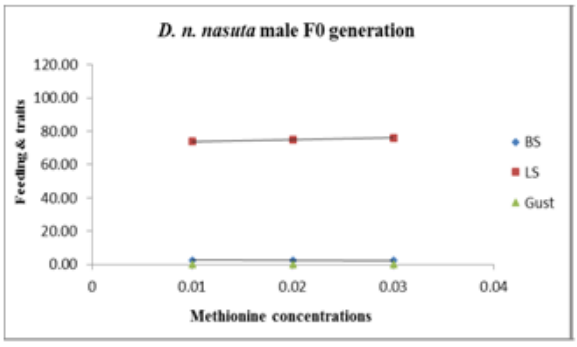

Figure 11

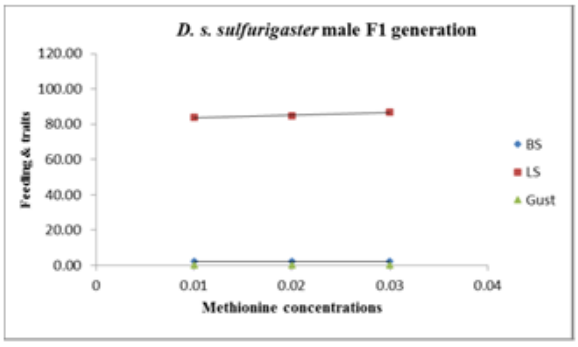

Figure 12

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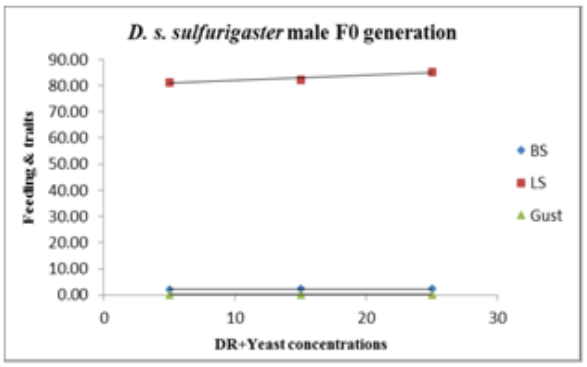

Figure 13

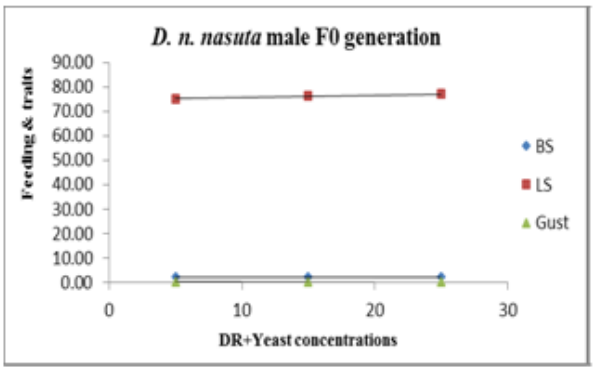

Figure 14

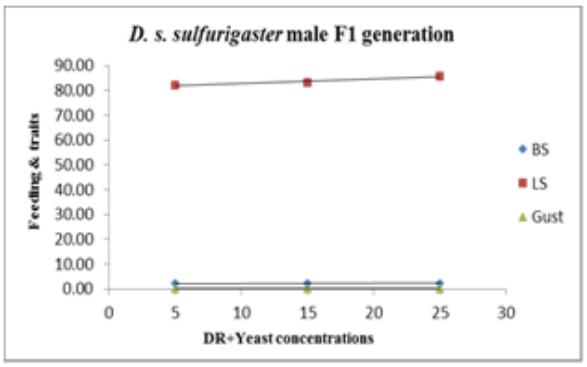

Figure 15

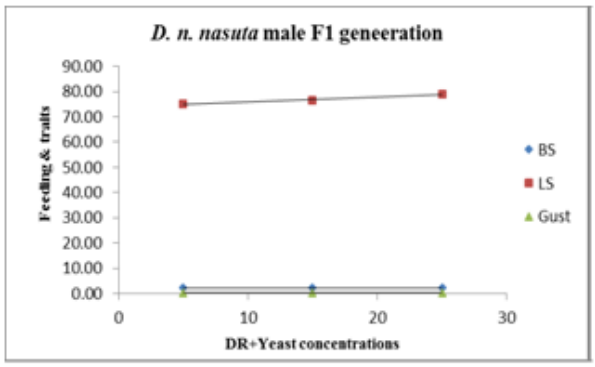

Figure 16

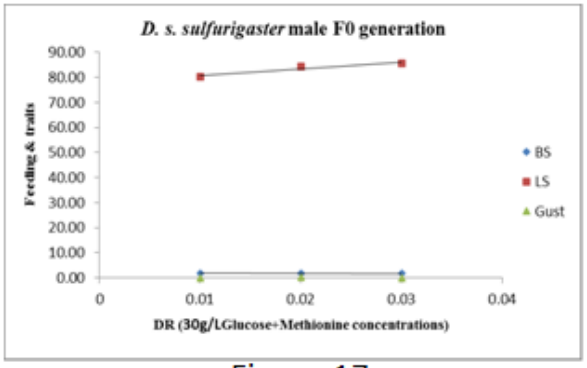

Figure 17

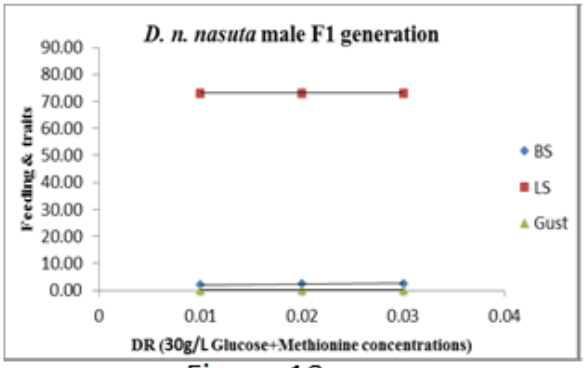

Figure 18

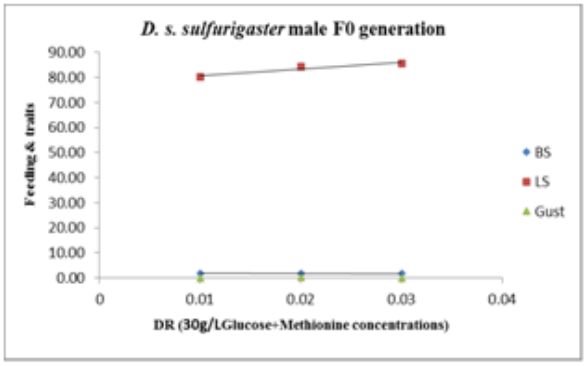

Figure 19

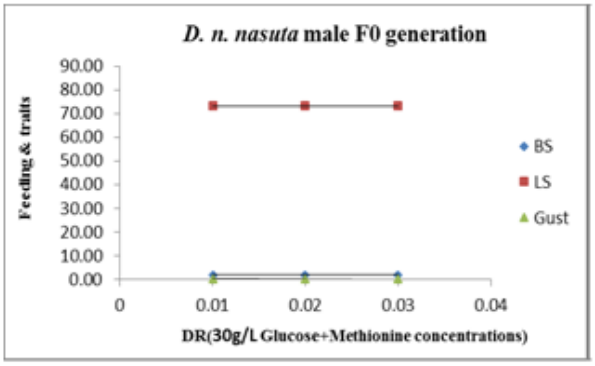

Figure 20

Figure.1-20. Genetic Correlation between dietary concentration with gustatory, metric traits (Body size) and life history traits between F0 and F1 generation in male flies of two species.

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Two-tailed P-values for the spearman's correlation non-linear coefficient.BS-Body size, LS-lifespan, Gust-Gustatory feeding.

In addition to this the metric traits (body size) and life history traits (fecundity and lifespan) of female flies are significantly correlated between them, When supplemented with single concentration of glucose and methionine showed positive correlation for fecundity and gustatory feeding in females with significant $p<0.01$ in both the species, and it is insignificant with body size and showed decrease in lifespan in both *D. n. nasuta*and *D.s.sulfurigaster*(F0 and F1 generation) **Table 1-2 and Table 3-4.**

In case of only yeast supplemented diet females showed negative correlation to fecundity as the diet concentration increased , and insignificant to body size in supplemented diet and showed r=945significant with lifespan in both F0 and F1 generation of *D. n. nasuta*and *D.s.sulfurigaster*. While in single concentration of methionine supplemented diet, females showed significant values for fecundity, gustatory feeding and body size, showing negative correlation with increase in the diet concentration in both *D. n. nasuta*and *D.s.sulfurigaster*species (F0 and F1 generation). And positive correlation to the lifespan in only F0 D. *n. nasuta*with significant value P<0.01significant r=0.01**Table 5-6**

In DR supplemented diet i.e. glucose along with yeast, and glucose with methionine the females showed positive correlation to the lifespan and negative correlation to fecundity, body size resulting in decrease in fecundity as the diet concentration increased withsignificant r=0.01 and insignificant between gustatory feeding and body size in the fed dietfor both *D. n. nasuta*and *D.s.sulfurigaster*species (F0 and F1 generation).**Table 7-8 and Table 9-10**

In consistence with these results the cost of reproduction has resulted with reduced lifespan, which implies the trade-off between varied diets and life history traits when supplemented with single concentration of glucose females have shown positive correlation with fecundity and negative correlation with lifespan and correlation is null with body size and gustatory feeding. Despite in single concentration of yeast and DR diet (30 g/L glucose + Yeast) it was vice versa in both species with generations. In methionine and mixed concentration i.e. DR diet (30 g/L glucose +methionine) the lifespan and fecundity were positively correlated and showing no correlation with gustatory and body size in both *D.n.nasuta*(F0 and F1 generation) and *D. s. sulfurigaster*(F0 and F1 generation).

Table 1:Genetic correlation between dietary supplements with gustatory, metric trait and life history traits in relation to glucose diet in *D. n. nasuta* female.

Traits ↓		Concentrations	Bodysize female F0	Bodysize female F1	Lifespan female F0	Lifespan female F1	Gustatory Feeding female F0	Gustatory Feeding female F1	Fecundity female F0	Fecundity female F1
Spearman's rho	Concentrations	1.000								
	Bodysize female F0	.189	1.000							
	Bodysize female F1	.189	1.000**	1.000						
	Lifespan female F0	-.794**	.343	.343	1.000					
	Lifespan female F1	-.680**	.514**	.514**	.968**	1.000				
	Gustatory Feeding female F0	-.580**	.482**	.482**	.941**	.941**	1.000			
	Gustatory Feeding female F1	-.351	.675**	.675**	.828**	.900**	.947**	1.000		
	Fecundity female F0	.945**	.436*	.436*	-.582**	-.429*	-.345	-.076	1.000	
	Fecundity female F1	.945**	.436*	.436*	-.582**	-.429*	-.345	-.076	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).*. Correlation is significant at the 0.05 level (2-tailed).

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Table 2: Genetic correlation between the dietary supplements with gustatory, metric traitand life history traits toyeast diet in *D. n. nasuta* female.

Traits ↓		Concentrations	Bodysize female F0	Bodysize female F1	Lifespan female F0	Lifespan female F1	Gustatory Feeding female F0	Gustatory Feeding female F1	Fecundity female F0	Fecundity female F1
Spearman's rho	Concentrations	1.000								
	Bodysize female F0	.303	1.000							
	Bodysize female F1	.303	1.000**	1.000						
	Lifespan female F0	.718**	.784**	.784**	1.000					
	Lifespan female F1	.718**	.784**	.784**	1.000**	1.000				
	Gustatory Feeding female F0	-.947**	-.039	-.039	-.471**	-.471**	1.000			
	Gustatory Feeding female F1	-.718**	.196	.196	-.110	-.110	.852**	1.000		
	Fecundity female F0	-.945**	-.027	-.027	-.479**	-.479**	.987**	.832**	1.000	
	Fecundity female F1	-.945**	-.027	-.027	-.479**	-.479**	.987**	.832**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

Table 3: Genetic correlation between the dietary supplementswith gustatory, metric traitand life history traits to methionine diet in *D. n. nasuta* female

Traits ↓		Concentrations	Bodysize female F0	Bodysize female F1	Lifespan female F0	Lifespan female F1	Gustatory Feeding female F0	Gustatory Feeding female F1	Fecundity female F0	Fecundity female F1
Spearman's rho	Concentrations	1.000								
	Bodysize female F0	-.094	1.000							
	Bodysize female F1	-.094	1.000**	1.000						
	Lifespan female F0	.794**	.471**	.471**	1.000					
	Lifespan female F1	-.094	.711**	.711**	.400*	1.000				
	Gustatory Feeding female F0	-.939**	.360	.360	-.548**	.380*	1.000			
	Gustatory Feeding female F1	-.903**	.433*	.433*	-.471**	.394*	.986**	1.000		
	Fecundity female F0	-.397*	.482**	.482**	-.057	-.061	.471**	.561**	1.000	
	Fecundity female F1	-.397*	.482**	.482**	-.057	-.061	.471**	.561**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed)

Table 4: Genetic correlation between the dietary supplements with gustatory, metric traitand life history traits to DR diet (glucose+Yeast) in *D. n. nasuta*female

Triats ↓		Concentrations	Bodysize female F0	Bodysize female F1	Lifespan female F0	Lifespan female F1	Gustatory Feeding female F0	Gustatory Feeding female F1	Fecundity female F0	Fecundity female F1
Spearman's rho	Concentrations	1.000								
	Bodysize female F0	.359	1.000							
	Bodysize female F1	.350	.999**	1.000						
	Lifespan female F0	.794**	.796**	.794**	1.000					
	Lifespan female F1	.661**	.679**	.685**	.814**	1.000				
	Gustatory Feeding	-.474**	.102	.095	-.138	-.546**	1.000			

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	female F0 Gustatory Feeding female F1	-.474**	.048	.068	-.245	.260	-.346	1.000		
	Fecundity female F0	-.397*	.143	.155	-.164	.325	-.376*	.967**	1.000	
	Fecundity female F1	-.397*	.143	.155	-.164	.325	-.376*	.967**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

Table 5: Genetic correlation between the dietary supplementswith gustatory, metric traitand life history traits to DR diet (glucose+ methionine) in *D. n. nasuta*female.

	Traits ↓	Concentrations	Bodysize female F0	Bodysize female F1	Lifespan female F0	Lifespan female F1	Gustatory Feeding female F0	Gustatory Feeding female F1	Fecundity female F0	Fecundity female F1
Spearman's rho	Concentrations	1.000								
	Bodysize female F0	.170	1.000							
	Bodysize female F1	.246	.936**	1.000						
	Lifespan female F0	.756**	.689**	.650**	1.000					
	Lifespan female F1	.756**	.689**	.650**	1.000**	1.000				
	Gustatory Feeding female F0	-.903**	.190	.095	-.440*	-.440*	1.000			
	Gustatory Feeding female F1	-.903**	.190	.095	-.440*	-.440*	1.000**	1.000		
	Fecundity female F0	-.529**	.571**	.421*	.057	.057	.748**	.748**	1.000	
	Fecundity female F1	-.529**	.571**	.421*	.057	.057	.748**	.748**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

Table 6: Genetic correlation between the dietary supplements with gustatory, metric traitand life history traits to glucose diet in *D. s. sulfurigaster*female

	Traits ↓	Concentrations	Body size female F0	Body size female F1	Life span female F0	Life span female F1	Gustatory feeding female F0	Gustatory feeding female F1	Fecundity female F0	Fecundity female f1
Spearman's rho	Concentrations	1.000								
	Body size female F0	.094	1.000							
	Body size female F1	.094	1.000**	1.000						
	Life span female F0	-.926**	.239	.239	1.000					
	Life span female F1	-.926**	.239	.239	1.000**	1.000				
	Gustatory feeding female F0	-.499**	.551**	.551**	.705**	.705**	1.000			
	Gustatory feeding female F1	-.581**	.593**	.593**	.816**	.816**	.952**	1.000		
	Fecundity female F0	.794**	.536**	.536**	-.564**	-.564**	.009	-.071	1.000	
	Fecundity female f1	.794**	.536**	.536**	-.564**	-.564**	.009	-.071	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

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Table 7:Genetic correlation between the dietary supplementswith gustatory, metric traitand life history traits to yeast diet in *D. s. sulfurigaster*female

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Traits ↓		Concentrations	Body size female F0	Body size female F1	Life span female F0	Life span female F1	Gustatory feeding female F0	Gustatory feeding female F1	Fecundity female F0	Fecundity female f1
Spearman's rho	Concentrations	1.000								
	Body size female F0	.303	1.000							
	Body size female F1	.303	1.000**	1.000						
	Life span female F0	.945**	.566**	.566**	1.000					
	Life span female F1	.549**	.594**	.594**	.669**	1.000				
	Gustatory feeding female F0	-.947**	-.039	-.039	-.793**	-.377*	1.000			
	Gustatory feeding female F1	-.947**	-.039	-.039	-.793**	-.377*	1.000**	1.000		
	Fecundity female F0	-.945**	-.027	-.027	-.796**	-.383*	.987**	.987**	1.000	
	Fecundity female f1	-.945**	-.027	-.027	-.796**	-.383*	.987**	.987**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 8:Genetic correlation between the dietary supplements with gustatory, metric traitand life history traits to methionine diet in *D. s. sulfurigaster*female

Traits ↓		Concentrations	Body size female F0	Body size female F1	Life span female F0	Life span female F1	Gustatory feeding female F0	Gustatory feeding female F1	Fecundity female F0	Fecundity female f1
Spearman's rho	Concentrations	1.000								
	Body size female F0	-.094	1.000							
	Body size female F1	-.094	1.000**	1.000						
	Life span female F0	-.094	.786**	.786**	1.000					
	Life span female F1	.491**	.489**	.489**	.775**	1.000				
	Gustatory feeding female F0	-.830**	.449*	.449*	.315	-.319	1.000			
	Gustatory feeding female F1	-.939**	.360	.360	.335	-.310	.956**	1.000		
	Fecundity female F0	.472**	.346	.346	.689**	.961**	-.427*	-.360	1.000	
	Fecundity female f1	.472**	.346	.346	.689**	.961**	-.427*	-.360	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

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Table 9:Genetic correlation between the dietary supplements with gustatory, metric traitand life history traits to DR diet (glucose+yeast) in *D. s. sulfurigaster*female

Traits ↓		Concentrations	Body size female F0	Body size female F1	Life span female F0	Life span female F1	Gustatory feeding female F0	Gustatory feeding female F1	Fecundity female F0	Fecundity female f1
Spearman's rho	Concentrations	1.000								
	Body size female F0	.094	1.000							
	Body size female F1	.094	1.000**	1.000						
	Life span female F0	.786**	.593**	.593**	1.000					
	Life span female F1	.936**	.397*	.397*	.928**	1.000				
	Gustatory feeding female F0	-.619**	.577**	.577**	-.077	-.315	1.000			
	Gustatory feeding female F1	-.474**	.347	.347	.022	-.321	.540**	1.000		
	Fecundity female F0	-.643**	.464**	.464**	-.113	-.429*	.745**	.881**	1.000	
	Fecundity female f1	-.643**	.464**	.464**	-.113	-.429*	.745**	.881**	1.000**	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 10: Genetic correlation between the dietary supplementswith gustatory, metric traitand life history traits to DR diet (glucose+methionine) *in D. s. sulfurigaster*female

Traits ↓		Concentrations	Body size female F0	Body size female F1	Life span female F0	Life span female F1	Gustatory feeding female F0	Gustatory feeding female F1	Fecundity female F0	Fecundity female f1
Spearman's rho	Concentrations	1.000								
	Body size female F0	-.189	1.000							
	Body size female F1	-.189	1.000**	1.000						
	Life span female F0	.852**	.290	.290	1.000					
	Life span female F1	.852**	.290	.290	1.000**	1.000				
	Gustatory feeding female F0	.474**	.168	.168	.591**	.591**	1.000			
	Gustatory feeding female F1	.474**	.168	.168	.591**	.591**	1.000**	1.000		
	Fecundity female F0	-.643**	.561**	.561**	-.317	-.317	-.587**	-.587**	1.000	
	Fecundity female f1	-.643**	.561**	.561**	-.317	-.317	-.587**	-.587**	1.000*	1.000

**. Correlation is significant at the 0.01 level (2-tailed).

4. DISCUSSION

According to life-history theory, natural selection could be expected to favour parents that produce fewer but better provisioned offspring in response to cues indicative that offspring will experience nutritional stress (32). The effects of DR have been investigated for more than 70 years in various organisms. Although DR is known to extend the lifespan of a wide range of organisms, species-specific effects of DR restriction have also been recorded (33). The nutrients that an organism absorbs from its diet are essential for development, and determine how organisms can maximise their fitness (34).

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In contrast to this study *Drosophila* male flies when fed with varied concentration of diets, lifespan did not show correlation with body size and gustatory feeding when fed with single concentration of glucose diet and it was moderately correlated with the single concentration of yeast fed diet and DR diet i.e. glucose with yeast, and positively correlated with single concentration of methionine and DR diet i.e. glucose with methionine in F0 and F1 generation *of D.n.nasuta* and *D. s. sulfurigaster.* Wherein the differences are significantly high in both F0 and F1 generation of *D. s. sulfurigaster* than *D.n.nasuta*.

In holometabolous insects, alteration in diet quality during development has wide ranging effects upon many life history characteristics (35). The two major nutritive components of diet that contribute to development are proteins and carbohydrates. Proteins provide essential amino acids necessary for viability. Imbalances in dietary amino acids can have significant effects upon development and fitness and may underlie the effect of dietary restriction on lifespan (36) .

A consistent correlate of extension of lifespan by DR is a reduction in fecundity of females. For instance, in *C. elegans*, *Drosophila*, mouse and rat, extension of lifespan by DR is accompanied by a reduction in female fecundity (37). Reproduction itself often reduces lifespan (38), either because it uses nutrients that cannot be used in somatic repair and maintenance (39) or because it directly inflicts somatic damage (40). Fecundity (number of offspring produced) comprises one of the most energetically expensive processes involved in reproduction and usually is taken as a proxy value for the total reproductive efforts (41). For invertebrate animals changes in fecundity due to dietary effects have been recorded for different systems and taxa including changes associated with food limitation (42), moisture content in the diet, specific nutrient deficiency (43), diet composition (44) and presence of inhibitory secondary metabolites (45). In insects, restricted access to dietary protein during the growth and development phase of the life cycle typically results in reduced body size (46).

In consistence with these results the cost of reproduction has resulted with reduced lifespan, which implies the trade-off between varied diets and life history traits when supplemented with single concentration of glucose females have shown positive correlation with fecundity and negative correlation with lifespan and correlation is null with body size and gustatory feeding. Despite in single concentration of yeast and DR diet (30 g/L glucose + Yeast) it was vice versa in both species with generations. In methionine and mixed concentration i.e. DR diet (30 g/L glucose +methionine) the lifespan and fecundity were positively correlated and showing no correlation with gustatory and body size in both *D.n.nasuta*(F0 and F1 generation) and *D. s. sulfurigaster*(F0 and F1 generation).

The lifespan has prolonged on supplementation with increased concentration of methionine diet. *D.s.sulfurigaster*has accomplished better than *D.n.nasuta* in both F0 and F1 generation. Among the different types of macronutrient restriction, reduced intake of proteins and increased concentration of single essential amino acid i.e. methionine fed has led to increased lifespan. However the single and combinatorial components of DR diet i.e. glucose with yeast and methionine act concertedly to enhance the fitness of the species, diet concentrations did not suffice all the life history traits. While compare to Control flies showing lower fitness values than the dieatary supplements to the species for successive generation. Thereby the cost of reproduction is reduced in control in comparison with treated flies, which clearly

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denotes the requirement of varied diet and concentrations are essential to enhance the fitness traits. Hence the enriched media with DR prerequisites are essential for the betterment of an organism.

5. CONCLUSION

The fitness benefits of developing to adulthood fast and those of being large often trade off with each other. Because in invertebrates, including *D. melanogaster*, body size is often positively correlated with female fecundity and male mating success.In contrast to this study *Drosophila* male flies when fed with varied concentration of diets, lifespan did not show correlation with body size and gustatory feeding when fed with single concentration of glucose diet and it was moderately correlated with the single concentration of yeast fed diet and DR diet i.e. glucose with yeast, and positively correlated with single concentration of methionine and DR diet i.e. glucose with methionine in F0 and F1 generation *of D.n.nasuta*and *D. s. sulfurigaster.* Wherein the differences are significantly high in both F0 and F1 generation of *D. s. sulfurigaster* than *D.n.nasuta*.Thus the results obtained has enhanced the evolutionary potential of species which selects for varied concentrations of dietary intake of carbohydrates, proteins and amino acids and has determine the cohesive fitness of life history traits.

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SYNTHESIS AND CHARACTERIZATION OF *2-SUBSTITUTED-4-(NAPHTHO [2,1-B]FURAN-2-YL)-2,3-DIHYDROBENZO[B][1,4]THIAZEPINE* FOR ANTIBACTERIAL ACTIVITY

Raghavendra S. M.[1,2], LathaK. P.[2*],Sanjay K. R.[3] and Sudarshan B L.[3]
[1]Department of Chemistry, Government First Grade College, Kadur-577 548, India
[2]Department of Chemistry, Sahyadri Science College, Shivamogga,-577203, India
[3]Department of Biotechnology, JSS Science and Technology University, Sri Jayachamarajendra College of Engineering Mysore, India

ABSTRACT: A series of 2-substituted-4-(naphtho[2,1-*b*]furan-2-yl)-2,3-dihydrobenzo[*b*][1,4]thiazepine **6(a-h)** were designed and synthesized. The substitutions are aliphatic, aromatic and heteroaromatic compounds. All the synthesized derivatives were characterized by using IR, Mass Spectral and H^1-NMR Spectral studies. All the series of molecules were screened for their diverse antibacterial activity in relative percent inhibition, minimum inhibitory concentration and minimum bactericidal concentration. All compounds in this series **6(a-h)** showed potent antibacterial activities.

KEYWORDS: Naphtho[2,1-*b*]furan, antibacterial activity, minimum inhibitory concentrations, minimum bactericidal concentration.

Corresponding author: Dr. LathaK. P, Department of Chemistry, Sahyadri Science College, Shivamogga,-577203, India

1. INTRODUCTION

Heterocyclic compounds play a vital role in organic chemistry, especially in the field of medicinal chemistry. More than half of the naturally occurring compounds and a high proportion of drugs contain heterocycles. Organic and medicinal chemistry is becoming very essential chemistry, explores the role of chemists towards isolation, characterization and synthesis of new compounds that can be used as medicine for the prevention, treatment and cure of certain diseases.

Amongst the various oxygen heterocycles, five membered heterocyclic systems, i.e., furan have been investigated to large extent. Furan ring when fused with carbocyclic systems such as benzene and naphthalene gives condensed heterocycles like, benzofurans and naphthofurans. Naphthofurans possess a broad range of biological activities, which are the constituents of important natural products [1-3].The literature survey revealed that derivatives of naphtho[2,1-b]furan synthesized have been found to exhibit a wide range of biological and pharmacological activity [4-7].

The complexes of naphtho[2,1-b] furan exhibit a wide range of biological activities including antimicrobial, anthelmintic, analgesic and anti-inflammatory activities [8].The derivatives of naphtho[2,1-b]furan synthesized have been found to possess wide spectrum of pharmacological and biological activities [9-10]. It is general observation that, introduction of nitro group sometimes enhances biological profile of the compounds to some extent. The nitro derivatives of naphtho[2,1-b]furan have been reported to exhibit antipyretic [11], analgesic [12], antihypertensive [13], antiviral [14], anti-inflammatory [15], antiparasitic [16], antimicrobial [17], anticancer activity [18] and some were shown to be mutagenic in bacteria [19]. Hence the literature survey reveals that, varieties of naphtho[2,1-b]furan derivatives exhibit prominent pharmacological and biological activity. This prompted us to undertake the synthesis of naphtho[2,1-*b*]furan derivatives **(Scheme-1)** and to evaluate the

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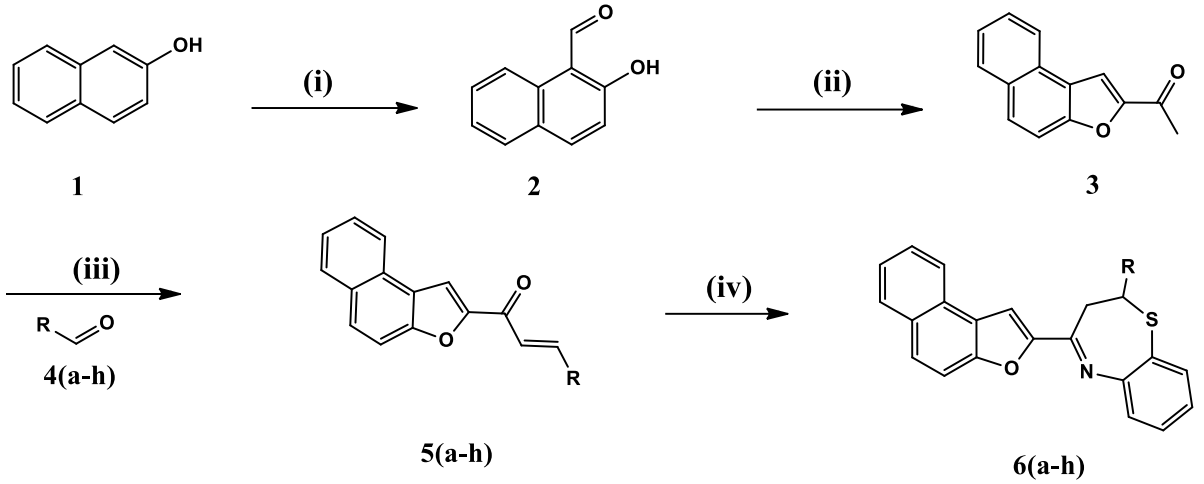
biological and pharmacological activities.



Scheme-1

Reagents : (i) $CHCl_3$, NaOH, **(ii)** K_2CO_3, $ClCH_2COCH_3$, **(iii)** EtOH, KOH, **4(a-h)** Aldehydes:(**4a**:Formaldehyde, **4b**:Benzaldehyde, **4c**:4-methylbenzaldehyde, **4d**:4-methoxybenzaldehyde, **4e**:4-ethoxybenzaldehyde, **4f**:4-fluorobenzaldehyde, **4g**:3-hydroxy-4-methoxybenzaldehyde, **4h**:picolinaldehyde), **(iv)** 2-aminothiophenol, C_2H_5OH, Piperidine, CH_3COOH

2. MATERIALS AND METHODS

2.1 CHEMISTRY

2.1.1 Materials and Methods: Infrared(IR) spectra were recorded using a Shimadzu Fourier transform infrared spectroscopy (FTIR)series. Melting points were determined using SELACO-650 hot stage melting point apparatus and were uncorrected. Mass and purity were recorded on a LCMSD-Trap-XCT. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Shimadzu AMX 400-Bruker, 400 MHz spectrometer using DMSO-d_6 as a solvent and TMS as internal standard (chemical shift in δ ppm). Spin multiplets were given as s (singlet), d (doublet), t (triplet) and m (multiplet). Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck made TLC plates.

2.1.2 Synthesis of 2-hydroxy-1-naphthaldehyde (2): naphthalen-2-ol (7 g, 0.1 mol), sodium hydroxide (23 g) dissolved in 12 ml water and 12 ml ethyl alcohol in a round bottom flask and was heated to 70–75°C.Then the Chloroform (7 ml, 0.15 mol) was added dropwise and the refluxed to 45 min.The mixture was refluxed further at 75–80 °C for a 1 h. and then allowed the reaction mixture room temperature. The reaction mixture was stirred for 3h in room temperature. The solid that separates out was filtered offand washed with ethyl alcohol. It was dissolved in the minimumamount of water and then acidified with dilute HCl. Solid which separatesout was washed with water and recrystallized from ethyl alcohol.Yield 63%, m.p. 81 °C. (literature m.p. 80 °C).

2.1.3 Synthesis of 1-(naphtho[2,1-b]furan-2-yl)ethanone (3):

2-hydroxy-1-naphthaldehyde **(2)** (5.1 g, 0.03 mol), chloroacetone (2.77 g, 0.03 mol) and anhydrous potassium carbonate (41.4 g, 0.3 mol) were taken in a round bottom flask containing dry acetone. The reaction mixture was refluxed for 24 h. Then the reaction mixture was filtered off and potassium carbonate was washed with acetone. The fraction was evaporated using a rotary evaporator and the residue was purified by silica gel column chromatography which was recrystallized from ethanol to get 1-(naphtho[2,1-b]furan-2-yl)ethanone **(3)**.

2.1.4 Synthesis of 1-(naphtho[2,1-b]furan-2-yl)-3-substituted-prop-2-en-1-one

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derivatives 5(a-h):

In a round bottom flask 1-(naphtho[2,1-b]furan-2-yl)ethanone **(3)** (0.004 mol) was dissolved in hot mixture of ethanol (100 ml) and sodium hydroxide (1 g). The reaction mixture was stirred for 10 minutes. Then, to the reaction mixture the different aliphatic, substituted aromatic and heterocyclic aldehydes (0.004 mol) were added. The reaction mixture was stirred for 12 h at 50-55 °C. The reaction mixture was washed with water and the product was extracted with ethyl acetate and treated with sodiumbisulphite. The solvent was evaporated using rotary evaporator and the obtained intermediates were purified by chromatographic column to get 1-(naphtho[2,1-b]furan-2-yl)-3-substituted-prop-2-en-1-one derivatives **5(a-h).**

2.1.5 Synthesis of 4-(naphtho[2,1-b]furan-2-yl)-2-substituted-2,3-dihydrobenzo[b][1,4] thiazepine derivatives 6(a-h):

Mixture of 1-(naphtho[2,1-b]furan-2-yl)-3-substituted-prop-2-en-1-one derivatives **5(a-h)** (0.01moles) and *O*-amino thiophenol (0.11 moles) were taken in round bottom flask containing 50 ml alcohol. To this reaction mixture, 3 ml of piperidine was added. Then the reaction mixture was refluxed for 6 h. The reaction mixture was acidified by 10 ml glacial acetic acid and continued for refflux for another 2 h. The reaction was monitored by TLC. Then the reaction mixture was allowed to cool to room temperature. A solid compound was obtained and filtered off, washed and recrystallized from aqueous acetic acid to get respective 4-(naphtho[2,1-b]furan-2-yl)-2-substituted-2,3-dihydrobenzo[b][1,4] thiazepine derivatives **6(a-h).**

2.1.5.1 2-methyl-4-(naphtho[2,1-b]furan-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepine(6a): $C_{20}H_{17}NOS$;M.P:255°C ; ^{1}H NMR (400 MHz, $CDCl_3$): □□1.32 (d, 3H, methyl),1.57(d, 1H, methylene), 1.88(d, 1H, methylene), 2.62(t, 1H, methylene), 6.65-7.72(complex m, 11H Ar. Proton)

2.1.5.2 4-(naphtho[2,1-b]furan-2-yl)-2-phenyl-2,3-dihydrobenzo[b][1,4]thiazepine(6b): $C_{27}H_{19}NOS$; M.P:241°C; ^{1}H NMR (400 MHz, $CDCl_3$):□□1.81(d, 1H, methylene), 2.20(d, 1H, methylene), 3.66 (t, 1H, methylene), 6.78-7.75 (complex m, 16H, Ar. Proton)

2.1.5.3 4-(naphtho[2,1-b]furan-2-yl)-2-(p-tolyl)-2,3-dihydrobenzo[b][1,4]thiazepine(6c): $C_{28}H_{21}NOS$; M.P:246°C^{1}H NMR (400 MHz, $CDCl_3$):□□1.82 (d, 1H, methylene),1.87(s, 3H, Ar-methyl), 2.22(d, 1H, methylene), 3.62(t, 1H, methylene), 6.78-7.75(complex m, 15H, Ar. Proton)

2.1.5.4 2-(4-methoxyphenyl)-4-(naphtho[2,1-b]furan-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepine (6d): $C_{28}H_{21}NO_2S$; M.P:268°C^{1}H NMR (400 MHz, $CDCl_3$):□□1.85 (d, 1H, methylene),1.91(d, 1H, methylene), 3.91(d, 3H, methoxy), 3.72(t, 1H, methylene), 6.68-7.73 (complex m, 15H, Ar. Proton)

2.1.5.5 2-(4-ethoxyphenyl)-4-(naphtho[2,1-b]furan-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepine (6e): $C_{29}H_{23}NO_2S$; M.P:271°C^{1}H NMR (400 MHz, $CDCl_3$):□□1.21(t, 3H, methyl), 1.86 (d, 1H, methylene),1.92(d, 1H, methylene), 3.75(t, 1H, methylene), 4.09(q, 2H, O-methyl), 6.65-7.73(complex m, 15H, Ar. Proton)

2.1.5.6 2-(4-fluorophenyl)-4-(naphtho[2,1-b]furan-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepine (6f): $C_{27}H_{18}FNOS$; M.P:290°C^{1}H NMR (400 MHz, $CDCl_3$):□□1.82 (d, 1H, methylene),2.22(d, 1H, methylene), 3.62(t, 1H, methylene), 7.28-8.75(complex m, 15H, Ar. Proton)

2.1.5.7 2-methoxy-5-(4-(naphtho[2,1-b]furan-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepin-2-yl)phenol (6g): $C_{28}H_{21}NO_3S$; M.P:296°C^{1}H NMR (400 MHz, $CDCl_3$):□□1.86 (d, 1H, methylene),1.99(d, 1H, methylene), 3.96(s, 3H, methoxy), 3.72(t, 1H, methylene), 6.69-7.76(complex m, 14H, Ar. Proton)

2.1.5.8 4-(naphtho[2,1-b]furan-2-yl)-2-(pyridin-2-yl)-2,3-dihydrobenzo[b][1,4]thiazepine(6h): $C_{26}H_{18}N_2OS$; M.P:269°C^{1}H NMR (400 MHz,

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$CDCl_3$):□□1.84(d, 1H, methylene), 2.02(d, 1H, methylene), 3.67(t, 1H, methylene), 6.78-7.85 (complex m, 15H, Ar. Proton)

2.2 BIOLOGY

2.2.1 Materials and Methods:

Microbial strains were procured From Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. Standard antibiotics & mediums were purchased from Sigma-Aldrich. All other chemicals were of analytical grade.

2.2.2 Determination of Relative Percentage Inhibition:

Synthesized eight compounds of 4-(naphtho[2,1-b]furan-2-yl)-2-substituted-2,3-dihydrobenzo[b][1,4] thiazepine derivatives were evaluated for antibacterial activity by agar diffusion method [20-21]. with slight modifications. For preliminary check and to select the efficient compound against two strains of gram positive bacteria (*Staphylococcus aureus* MTCC-7443 and *Baccilus licheniformis* MTCC-2465), Two strains of gram negative bacteria (*Escherichia coli*, and *Acetobacter sp*., MTTC-3245). The inoculum was adjusted to approximately 2×10^5 CFU/ml with sterile saline solution. All synthesized compounds were dissolved 10 mg/mL in dimethyl sulfoxide (DMSO) as a stock solution and loaded different concentration ranges 100 µg, 200 µg, 300 µg 400 µg and 500 µg for different wells. The medium used were nutrient agar, DMSO as negative control and as positive control standard antibiotics Rifampicin (5 u/disc) & Bacitracin (10 u/disc) were used for bacteria. After 18 h of incubation at 37 ^{0}C the diameter of inhibition zone (mm) was measured and relative percent inhibition was calculated **(Table 1).**

Table 1: Calculated Relative percent inhibition of the synthesized molecules

	Relative per cent inhibition			
Compound No	**Gram – ve Bacteria**		**Gram + ve Bacteria**	
	Escherichia coli (Ec)	**Acetobacter sp. (As)**	**Baccilus licheniformics (Bl)**	**Staphylococcus aureus (Sa)**
6a	53 ± 8.2	36 ± 6.2	23 ± 5.3	15 ± 3.7
6b	37 ± 6.8	24 ± 4.9	18 ± 4.5	27 ± 5.4
6c	48 ± 5.7	37 ± 8.2	21 ± 7.7	32 ± 6.7
6d	82 ± 11.4	73 ± 9.4	51 ± 10.4	63 ± 14.5
6e	79 ± 13.6	95 ± 9.6	66 ± 11.2	52 ± 11.2
6f	38 ± 5.2	25 ± 7.2	48 ± 7.2	26 ± 3.7
6g	76 ± 11.3	61 ± 8.3	86 ± 9.3	78 ± 13.4
6h	24 ± 8.4	32 ± 4.9	34 ± 9.2	14 ± 3.2

2.2.3 Determination of MIC and MBC:

Based on the previous agar diffusion method, eight compounds **6 (a-h)** were determined for minimum inhibitory concentrations (MIC) by broth dilution method [22] modified with the addition of 0.15 % agar [23-25]. From the Stock solutions the compounds were serially diluted (0, to 100 µg/ml (w/v)) in DMSO was dispensed into rows of wells in micro titre plates (48 wells). Equal volume of inoculum (2×10^5 CFU/ml) was added into appropriate wells and mixed with the growth medium and incubated at 37 °C for 48 h. The growth and MIC values were determined as the lowest concentration that inhibits the growth of microorganisms. Similarly minimum bactericidal concentration (MBC) values were determined as the lowest concentration that completely kills bacterial cells (**Table 2**).

2.2.4 Statistical analysis: Data of all experiments were conducted in triplicates and expressed as mean ± standard deviation of three trials. The relative percentage inhibition with

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respect to positive and negative control was calculated by the familiar formula as fallows.
Relative percentage inhibition of the compound = [{100 x (a - b)}/(c - b)]
Where, 'a' is total inhibition area of the test extract.
'b' is total inhibition area of the solvent
'c' is total inhibition area of the standard drug.

Table 2: Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentration (MBC) values of the synthesized molecules

Compound No.	MIC µg/ml				MBC µg/ml			
	Gram – ve Bacteria		Gram + ve Bacteria		Gram – ve Bacteria		Gram + ve Bacteria	
	Ec	As	Bl	Sa	Ec	As	Bl	Sa
6a	12 ± 0.5	13 ± 0.5	12 ± 0.5	13 ± 0.5	50 ± 10	70 ± 8	40 ± 5	60 ± 8
6b	30 ± 1.5	32 ± 1.1	36 ± 1.5	34 ± 1.0	180 ± 15	160 ±10	210 ± 15	220 ± 17
6c	15 ± 1.1	23 ± 0.5	23 ± 0.5	24 ± 0.5	80 ± 15	80 ± 8	90 ± 10	80 ± 10
6d	7 ± 0.5	10 ± 1.0	16 ± 1.0	15 ± 1.1	67 ± 15	54 ± 15	48 ± 17	60 ± 20
6e	13 ± 0.5	12 ± 0.5	12 ± 0.5	14 ± 0.5	40 ± 5	50 ±10	30 ± 5	60 ± 8
6f	32 ± 0.5	29 ± 0.5	32 ± 0.5	32 ± 0.5	140 ± 6	140 ±6	130 ± 5	140 ± 6
6g	13 ± 0.5	14 ± 1.0	14 ± 0.5	15 ± 0.5	80 ± 10	70 ± 8	80 ± 6	100 ± 11
6h	44 ± 0.5	44 ± 0.5	42 ± 0.5	42 ± 0.5	220 ± 11	217 ±15	200 ± 8	226 ± 10

3. RESULTS AND DISCUSSION

3.1 Chemistry: 2-substituted-4-(naphtho[2,1-*b*]furan-2-yl)-2,3-dihydrobenzo[*b*][1,4]thiazepine **6(a-h)** derivatives were synthesized using naphthalen-2-ol as a starting material and converted to 2-hydroxy-1-naphthaldehyde **(2)** via Riemier-Tiemenn reaction. The 2-hydroxy-1-naphthaldehyde **(2)** reacts with chloroacetate and anhydrous potassium carbonate to give 1-(naphtho[2,1-b]furan-2-yl)ethanone **(3)** which reacts with ethanol, NaOH and different aliphatic, substituted aromatic and heterocyclic aldehydes to give a series of intermediates (*E*)-1-(naphtho[2,1-b]furan-2-yl)-3-substituted-prop-2-en-1-one derivatives **5(a-h)** fallowed by cyclization with *O*-amino thiophenol to get 4-(naphtho[2,1-b]furan-2-yl)-2-substituted-2,3-dihydrobenzo[b][1,4] thiazepine derivatives **6(a-h).**

3.2 Biology: The antibacterial activity of synthesized compounds **6(a-h)** were evaluated and compared with Rifampicin and Bacitracin as standard drug. All eight compounds were showed good antibacterial properties against four human pathogenic bacterial strains which includes gram +ve and gram –ve species. Among eight compounds, 6a, 6d, 6e and 6g compounds were found to be potent inhibition to gram positive and the same trend was exhibited for gram negative species but comparatively it was lesser inhibition in gram negative species. Further, all eight compounds were evaluated for minimum inhibition capacity and minimum bactericidal efficacy for the above same bacterial strains. Here also, same trend was exhibited i.e., compounds, 6a, 6d, 6e and 6g were shown lesser concentration of compounds to inhibit minimum amount of cells similarly lesser concentration showed for MBC. These observations are due to the different electron withdrawing and electron donating groups attached to phenyl ring in compounds 6a, 6d, 6e and 6g.

4. CONCLUSION

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A series of 2-substituted-4-(naphtho[2,1-*b*]furan-2-yl)-2,3-dihydrobenzo[*b*] [1,4] thiazepine **6(a-h)** derivatives were synthesized according to the above synthetic methods. All the molecules were screened for their antibacterial properties against four human pathogenic strains including gram positive and gram negative. Fewer compounds in the series showed potent inhibition against all the four human pathogenic bacterial strains and they could be applicable for antibacterial drug development.

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PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITIES IN TWO SPECIES OF WILD ONIONS

M.Smitharaj, M.N. Shiva Kameshwari
Department of Botany, Bangalore University, Jnanabharathi Campus, Bangalore (Karnataka)-India

ABSTRACT: Wild onions are used in the medicinal system due to its high bio-efficacy.In the present investigation,it was aimed to evaluate the biopotentials of *Urginea indica* and *Urginea wightii*. antioxidant activity was estimated in two species of *Urginea* that is *Urginea indica* and *Urginea wightii* using three different assays DPPH, ABTS and Nitric oxide assays. *Urginea wightii* showed more activity in DPPH with 64.68 µg/ml against standard quercetin IC_{50} value 4. 047 µg/ml. In ABTS assay *Urginea indica* showed more activity with 208 µg/ml against standard quercetin IC_{50} value 1.552 µg/ml. In Nitric oxide assay both the species responded well to the activity with value 371.9 µg/ml in *Urginea wightii* while in *Urginea indica* 174.1 µg/ml against standard curcuminoids IC_{50} value 28.67 µg/ml. The compounds responsible for the antioxidant activity in *Urginea wightii* is alkaloids and terpenoids and in *Urginea indica* flavanoids and alkaloids have played a significant role in enhancing antioxidant activity.

KEYWORDS:*Urginea indica,Urginea wightii*, DPPH, ABTS, Nitric oxide, Alkaloid, Flavanoid, Terpenoid

1. INTRODUCTION

Natural antioxidants are the secondary metabolites of plants. Several classes of antioxidants are present in various dietary supplements and have been suggested for the health benefits. Consumption of these products leds to the reduction of various inflammatory and oxidative stress biomarkers [1]. Plants produce a very impressive array of antioxidant compounds that includes carotenoids, flavonoids, cinnamic acids, benzoic acids, folic acid, ascorbic acid, tocopherols and tocotrienols to prevent oxidation of the susceptible substrate [2]. Antioxidants are secondary constituents or metabolites found naturally in the body and in plants such as fruits and vegetables. An antioxidant can be defined in simple terms as anything that inhibits or prevents oxidation of a susceptible substrate. During metabolism, oxygen consumption involves the constant generation of free radicals and reactive oxygen species (ROS). H_2O_2 and O_2 can interact in the presence of certain transition metal ions to yield a highly- reactive oxidizing species, the hydroxyl radical (OH) [3] . Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species otherwise called, free radicals such as singlet oxygen, super oxide anion, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage[4] . Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischema, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing[5-6] .The antioxidants act by reacting with free oxygen radicals. The antioxidant is one of the essential substances which can protect the body from free radicals generated due to oxidative stress or various physiological functions. To lower the risk of various degenerative diseases, studies on evaluating the antioxidant potential of different plants and dietary supplements is extensively studied [7]. Nowadays antioxidants have motivated researchers' interest in both medicinal plants and synthetic organic compounds [8]. The implication of free radicals and reactive oxygen species (ROS) has been found to be in the pathogenicity of numerous diseases, including various chronic diseases [9-10]. Antioxidants are recently invented as the drug candidates to counter these diverse diseases,

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such as carcinogenesis, inflammation, and aging in aerobic organisms [11]. The design of small molecular agents to conflict cellular oxidative stress has become an important therapeutic objective, towards comprehensive damage to cellular macromolecules caused by reactive oxygen species (ROS) [12]. The extensive action of synthetic antioxidants is being ruled out owing to their toxicity and unwanted side effects and there is a growing interest in the use of the natural product as antioxidants and their derivatives for the treatment of oxidative stress-related diseases [13].

The genus Urginea is commonly called as 'Squill', which has an ancient and more or less honourable history as a medicinal plant.*Urginea indica* is rare and threatened Indian medicinal plant belongs to Hyacinthaceae family. *Urginea indica* is glabrous herb with a pale, ovoid or pear-shaped bulb which is 5 to 10 cm long, it grows in Salt Range, Kotli Near Mirpur and Mt. Tilla, where it commonly known as "Indian squill" and locally in Pakistan as "Junglipiyaz" [14]. Indian squill is considered to have medicinal value and is largely used as an expectorant, cardiac stimulant, in treating rheumatism, dropsy, edema, gout, asthma and as an anticancer agent[15]. Squill bulbs have long been used as a source of natural product with pharmaceutical and biocidal applications[16] . It is a vital therapeutic bulb used to cure infectious injury [17]. These species were introduced in Australia and North America, where they have become invasive species. It enhances immune system activity like fever, cold, mouth and pharynx inflammation, infections. It also improves immune system activity like bronchitis/cough, hyper tension, dyspepsia and arterio-sclerosis[18-20] . Some varieties of squill have been known to be effective rodenticides for more than a thousand years. In humans, extracts of the bulb were used as a cardiotonic by the Romans and ancient Egyptians for the treatment of edema, as an expectorant, and as an emetic. It continues to find use as an expectorant in some commercial cold preparations. Because of the popularity of the digitalis glycosides, squill components are used in the United States as cardioactive agents. Dry skin of wild onion contains Quercetin it is utilized as a yellow dye, which is anti-allergic and is also helpful in treating inflammatory bowel disease[21]. It contains Sulphur compounds, carbohydrates, proteins, phenolic compounds, saponins, quercetin[22]. In the present study an attempt has been made to know the antioxidant activity in two species of *Urginea* using three different assays.

2. MATERIALS AND METHODS

2.1. Chemicals

ABTS (sigma, USA), PBS-Phosphate buffer saline, Quercetin.

DPPH (EEC No.217-591-8, sigma, USA), Methanol, HPLC grade(Ranbaxy), Quercetin.

Sodium nitroprusside, Sulfanilamide, curcuminoids, sodium chloride, Potassium dihydrogen orthophosphate, Di-potassium hydrogen orthophosphate, Methanol HPLC grade.

2.2. Plant material collection and preparation of the extract

The fresh and mature bulb of two different species of genus *Urginea* such as *Urginea indica* and *Urginea wightii* were collected from two different locations Udupi (Karnataka) and yediyur (Karnataka). The collected bulbs were cleaned and washed under running tap water; then the bulbs were cut in to small pieces followed by shade drying for four weeks.The dried fruits were powdered using mixer.A soxhlet apparatus was used for the extraction using solvent such as methanol.For extraction 50g of the ground bulb powder was loaded to the thimble,and 500 ml of the given solvent was added in to the flask and refluxed 18 h.To ensure the maximum extraction,the process was repeated twice.The obtained extracts were concentrated using a rotary evaporator under reduced pressure[23-25] and later extracts were dissolved in respective solvent prior and used for further analysis.

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2.3. Estimation of total alkaloid content

The total alkaloid content in methanolic extracts of *Urginea indica*, *Urginea wightii* bulbs were determined according to method employed by Singh *et al.* (2004)[26], using colchicine as standard. The total alkaloid content of the samples was measured using 1, 10-phenanthroline. The reaction mixture contained 1ml plant extract, 1ml of 0.025M Ferric chloride in 0.5M hydrochloric acid and 1ml of 0.05M of 1, 10-phenantroline in ethanol. The mixture was incubated for 30 minutes in hot water bath with maintained temperature of 70ºC. The absorbance of red coloured complex was measured at 510nm against reagent blank.

2.4. Estimation of flavonoid content

Aluminium chloride colorimetric method was used to determine the flavonoid content as described by Chang *et al.,* (200)[27]. One milliliter of plant extracts was mixed with 0.5ml of aluminum chloride (1.2%) and 0.5ml of 120 mM potassium acetate. The mixture was allowed to stand for 30 minutes at room temperature. The absorbance was measured at 415nm and flavonoid content was expressed in terms of Rutin equivalent.

2.5. Estimation of terpenoid content

The terpenoid was determined calorimetrically by using linalool as standard. To 1ml of plant extracts, 1.5ml of chloroform was added and incubated for 5 minutes. Later, 0.5ml of sulphuric acid was added and cooled for 15 minutes. The mixture was incubated at room temperature for 1-2 hr in dark condition and colour changed to reddish brown to which 95% methanol and 5% distilled water was added. The absorbance was measured at 538nm.The standard used was linalool.

2.6 Assessment of antioxidant activity

2.6.1 DPPH Radical Scavenging Activity

DPPH assay is carried out as per the method of Rajakumar *et al*. (1994)[28] In brief, 75 µl of DPPH solution; various concentration of test solution and quantity sufficient to 3 ml with HPLC grade methanol. The different concentrations tested for REFERENCES standard in ug/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510 nm using semi-autoanalyzer. A control reaction is carried out without the test sample.

Calculating percentage growth inhibition:

$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ test\ Sample)}{OD\ of\ Control} \times 100$$

2.6.2 ABTS radical scavenging activity

The assay is performed as per Auddy *et al*.,(2003) [29] ABTS radical cations are produced by reacting ABTS and APS on incubating the mixture at room temperature in dark for 16 hours. The solution thus obtained is further diluted with PBS to give an absorbance of 1.000. Different concentrations of the test sample and the REFERENCES standard (highest volume taken was 50µl) are added to 950 µl of ABTS working solution to give a final volume of 1ml, made up by adding PBS. The absorbance is recorded immediately at 734nm. The percent inhibition is calculated at different concentrations and the IC_{50} values are calculated by Log-Probit analysis.

Calculating percentage growth inhibition:

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$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ test\ Sample)}{OD\ of\ Control} \times 100$$

2.6.3 NO Radical scavenging activity

Nitrogen oxide radical scavenging assay was measured spectrophotometrically [30]. In this assay 200µl of 10 mM sodium nitroprusside and 200 µl of test solution / REFERENCES standard of various concentrations are incubated at room temperature for 150 minutes. Add 500 µl Griess reagent and incubate for 10 minutes at room temperature. Measure the absorbance at 546 nm. Test substances are replaced by buffer solution for a control. The percent inhibition is calculated at different concentrations and the IC_{50} values are calculated by Log-Probit analysis.

Calculating percentage growth inhibition:

$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ test\ Sample)}{OD\ of\ Control} \times 100$$

3. RESULTS

3.1 Quantification of phytochemicals

The crude extract was subjected for chemical group tests to quantification various types of important chemical constituents. Phytochemical screening showed that alkaloids, flavonoids and terpenoids are present in the methanolic extract of *Urginea* species (Table 1).

The total alkaloid content was found to be maximum in *Urginea wightii* and *Urginea indica* (3.101± 0.056µg/ml and 2.897± 0.319µg/ml). Among the two species *Urginea indica* showed more flavonoid content that is 2.947± 0.389µg/ml compare to *Urginea wightii* that is 0.400 ± 0.144µg/ml. The higher concentration of terpenoid was obtained in *Urginea wightii* and recorded as 5.419± 1.054 µg/ml while *Urginea indica* showed 0.397± 0.078 µg/ml respectively.

Table-1: Quantification of alkaloid, flavonoid and terpenoid of methanolic extracts of *Urginea wightii* and *Urginea indica* (Udupi and Yediyur**)**

SI NO.	Sample	Alkaloid	Flavanoid	Terpenoid
1	*Urginea wightii(*Yediyur*)*	3.101±0.056	0.400 ± 0.144	5.419± 1.054
2	*Urginea indica(*Udupi)	2.897± 0.319	2.947± 0.389	0.397± 0.078

3.2 DPPH Radical Scavenging Activity

The antioxidant activity was determined by DPPH free radical scavenging assay where Quercetin was used as standard. In the present investigation Yediyur extract showed significant antioxidant activity with IC_{50} value 64.68µg/ml against standard Quercetin IC_{50} value 4.047 while *Urginea indica* did not show any activity to this assay.

Graph-1: DPPH radical scavenging activity in *Urginea wightii* (Yediyur)

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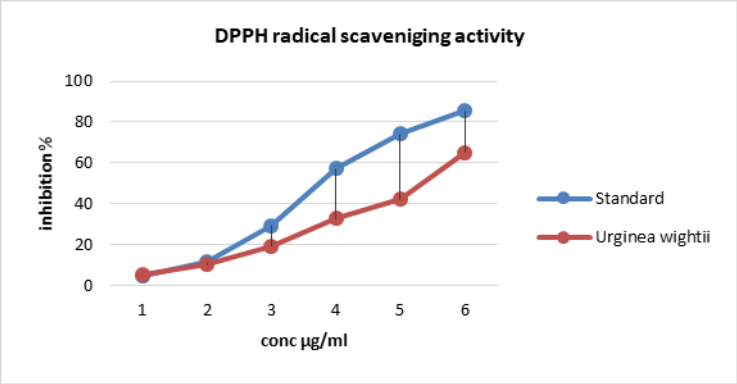
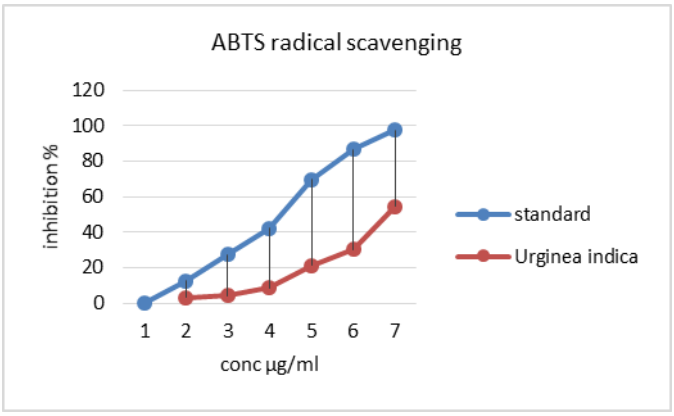


3.3 ABTS radical scavenging activity

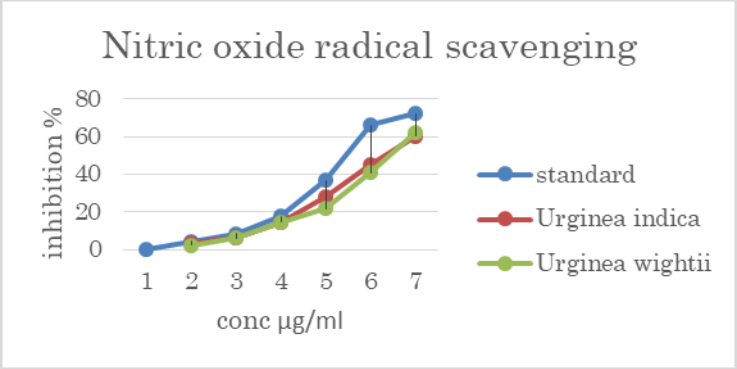
The antioxidant activity was determined by ABTS free radical scavenging assay where Quercetin was used as standard. In present investigation Quercetin showed significant antioxidant activity with IC_{50} value of 1.552 µg/ml. similarly *Urginea indica* showed significant antioxidant activity with IC_{50} value of 208 µg/ml. while *Urginea wightii* did not show any activity and less inhibition were noticed .



Graph-2: ABTS radical scavenging activity in *Urginea indica*(Udupi)

3.4 NO Radical scavenging activity.

The antioxidant activity was determined by nitric oxide free radical scavenging assay where curcuminoids was used as standard. In present investigation, Udupi and Yediyur extract showed significant antioxidant activity with IC_{50} value of 174.1 and 371.9µg/ml respectively against standard curcuminoids 28.67µg/ml.



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Graph-3: Nitric oxide radical scavenging activity in both *Urginea indica* and *Urginea wightii*

4. DISCUSSION

In the present investigation,preliminary results on the screening of phytochemicals revealed the presence of therapeutically essential constituents like alkaloid,flavonoid and terpenoid in extracts obtained from methanol solvent.

Quantitative estimation of *Urginea wightii* showed 5.419 µg/ml of terpenoids and 3.101 µg/ml alkaloids. Alkaloids or terpenoids might have played role in antioxidant activity of *Urginea wightii* yediyur accession and responded well to DPPH assay. While in ABTS assay *Urginea indica* (Udupi) showed more activity than *Urginea wightii*(Yediyur) and this is due to high alkaloid and flavonoids present in *Urginea indica*. In nitric oxide assay flavanoid, alkaloid and terpenoids of *Urginea indica* and *Urginea wightii* played significant role in showing highest antioxidant activity. Thus different assays result clearly revealed the antioxidant activity in both *Urginea indica* and *Urginea wightii* with little variations.

Antioxidants are potent candidates for the management and balance the average level of free radicals in the cells.DPPH is the compassionate and widely used method for evaluating the antioxidant potential of an extracts or purified compound.Intially,,DPPH was used to assesss the ability of *urginea* bulb extracts.*Urginea wightii* (Yediyur) showed more scavenging activity than *Urginea indica* in DPPH assay where as in ABTS assay no activity in *Urginea wightii* (Yediyur) but *Urginea indica* showed highest scavenging activity with 208 µg/ml.IC_{50} value.Therfore it can be concluded that methanolic extract of *Urginea indica* and *Urginea wightii* have significant level of antioxidant activity in nitric oxide assay followed by ABTS assay where only *Urginea indica* showed its scavenging activity to DPPH assay *Urginea wightii* responded little with only 64.68 µg/ml.Ic_{50} value. Further the phytocompounds identification responsible for these activities are correlated to terpenoids, alkaloid and flavonoids concentration.Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation,neuronal signaling,inhibition of platelet aggregation and regulation of cell mediated toxicity.It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger,vasodilation and antimicrobial and antitumour activities[31] Finally, to conclude the nitric oxide assay is very well suited to detect antioxidant activity in *Urginea* species since all the two plants responded and showed highest antioxidant activity to this assay.

5. CONCLUSION

The results of the present study indicate that the methanol extracts of *Urginea indica* and *Urginea wightii* bulb possess significant antioxidant potentials in dose dependant manner. The present data justify the traditional uses of this bulb for the treatment of various diseases. However, further studies are required for isolation and purification of the active principles of the plant responsible for these effects and to better understand the mechanism of such actions. We conclude from the above discussion that those methanolic extracts have antioxidant activity and phytochemicals by DPPH,ABTS and nitric oxide scavenging activity.It is very much helpful for investigation of bioactive compounds for various diseases by identifying the bioactive compound isolation process.

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FLOW CYTOMETRIC ANALYSIS OF GENOME SIZE ESTIMATIONS IN *URGINEA INDICA* AND *URGINEA WIGHTII*.

Ramya. M[1*], Shivakameshwari M. N.[2]
[1]School of Applied Sciences, REVA University, Kattigenahalli, Bengaluru – 64, India.
[2]Department of Botany, Bangalore University, Bengaluru – 56, India.

ABSTRACT: Angiosperms are well known for its diverse inclusion of flowering plants with around 17000 and odd species. Largest genome size of the angiosperms reported till date is 132.3 pg DNA (*Paris japonica)*. To estimate the content of the genome in the cell, initially feulgen densitometry technique was used. With the advent of flow cytometry, the analysis of genome content became more convenient. In the present study, the genome sizes of two different species of the *Urginea* species – *Urginea indica* (Nagarhole) and *Urginea wightii* (Gulbarga)were analysed to know the nuclear DNA content. The flow cytometric estimations were made following the technique employed by Sayanthani *et. al.,* 2014using the young root tips, macerated in modified Galbraith's buffer and stained with Propidium iodide. The prepared cell suspension was run in a BD FACS Calibur Flow Cytometer. Prior to sample run, the cytometer was calibrated and cell debris, doublets were excluded by gating the scatter profiles. Flow cytometry is a relative measure where an organism whose known stable genome size is used to estimate the unknown genome size. *Allium cepa*, with a known genome size of 2C = 33.5 pg DNA was used as standard. The histograms obtained were analyzed using Cellfit software and the G1 peak of the unknown sample was calculated using the quotient of sample G1 peak and standard G1 peak multiplied by the standard 2C DNA content. The genome size of *Urginea indica* (Nagarhole) was found to be 69.8 ± 0.29 pg - 34132.2 bp of DNA and *Urginea wightii* (Gulbarga) was found to have 55.21 ± 0.27 pg - 27002.5 of DNA. The variation in the genome sizes infers to the different amounts of non-coding DNA Intra-specific genome size variation is considered as a taxonomically informative tool for a complex genus *Urginea.*

Keywords: Intraspecific variation, genome size, flow cytometry, Propidium iodide.

***Corresponding author:** School of Applied Sciences, REVA University, Kattigenahalli, Bengaluru – 64, India. Email: ramya.m@reva.edu.in

1. INTRODUCTION

Urginea genus is known for its medicinal values, though not explored to a greater extent. It has gained its place in British and European pharmacopias due to its antiepileptic, cardiac stimulant and antipsoriatic properties. It is also used in the treatment of edema, allergies, male sterility and dropsy [1]. Surprisingly, the genus has not attracted greater attention of the researchers in India.

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Fig. 1: Vegetative and reproductive phases of *U. indica*

Fig. 2 : Vegetative and reproductive phases of *U. wightii*

Genome size or DNA C-value is an essential parameter in biology and biodiversity. C-values are increasingly useful in a phylogenetic study. Genome size data in combination with other characters can contribute to inter-generic classification, taxa delimitation, or hybrid identification. Genome size shows correlations with different phenotypic characters at the cell, tissue and organism levels but also to a broad range of external ecological issues and environmental concerns. Hence, the pattern of genome size variation can be considered as an important parameter to aid inter-generic and intra-generic delimitation of the family Hyacinthaceae. Using flow cytometry, there are only a few genome size estimates made in Hyacinthaceae, although there are many estimates reported in the genome size database, which have been estimated by Feulgen micro-densitometry. Optimization of buffer system was initially performed in *Urginea* indica and then the optimized buffer was used to estimate the nuclear DNA contents of these populations. C-values have been estimated for nearly 3500 species of angiosperms, representing over 1% of the approximately 250,000 – 300,000 species of flowering plants [2,3]. Despite this enormous range in DNA amount, the basic complement of genes required for normal growth and development appears to be essentially the same, leading to what is referred to as the ‘‘C-value paradox’’ [4]. Hewson Swift coined the term C-value but did not define it. The confusion created by the term was finally resolved by him, where he stated that the term was used to distinguish the diploid content and DNA amount. The letter C stood for ‘constant’ *i.e.*, the amount of DNA that was characteristic of a particular genotype. Ogur *et al.*, 1951 made the first genome size estimate in the plant *Lillium longiflorum* cv Croft. Changes in genome size facilitate evolutionary adaptation to environmental conditions; hence it has a significant impact on the evolution of plants and animals. It correlates with many organismal traits such as cell size, metabolism or growth rate. Flow cytometric analysis is difficult in plants because the preparation of suspensions of intact nuclei was comparatively tedious. In 1973, Heller performed the first successful experiment by preparing the suspensions of field bean nuclei from enzymatically treated fixed root tips. Ethidium bromide was used as a fluorescent dye to stain the nuclear DNA and using the emitted relative fluorescence intensity, the cell cycle kinetics was determined. But the technique stayed dormant for almost a decade because of its expensive instrumentation requirement. Nuclear DNA could be stained in plant protoplasts and fixation with ethanol–acetic acid decreases the auto-fluorescence[5].

Many researchers reported that the poor quality of histograms of cytometric estimations may

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be due to the 'off-centre' position of the nucleus [6,8,9]. In subsequent studies on the analysis, intact nuclei may be released from protoplasts by lysis either in the presence of a detergent or in a hypotonic medium lead to better histograms of DNA content [5,10,11]. The standard should be genetically stable with constant genome size, easy to use, and available in sufficient quantities. The genome size of the standard should be known with sufficient precision. As a result, different authors have used different standards, including human, domestic chicken and rainbow trout, as well as various plant species such as petunia, alfalfa and oats [12-17]. The standard material used was *Allium cepa,* with reported 2C-value of 33.5 pg of DNA. The nuclear DNA amount of *Allium cepa (*2C = 33.5 pg), obtained using chemical methods was later used as a calibration standard by Rees and colleagues at Aberystwyth, Cambridge and in Vienna [18-20]. From this literature background, the objective of the study was to determine the genome size in the species of *Urginea indica* and *Urginea wightii,* collected from Nagarhole and Gulbarga respectively.

2. MATERIALS AND METHODS

The bulbs of *Urginea* were collected from Nagarhole and Gulbarga. Voucher specimens of these bulbs have been deposited and maintained in the green house of Department of Botany, Bangalore University. These accessions were used for the genomic studies.

2.1 Isolation buffers

The chemical composition of the most popular nuclear isolation buffers used for flow cytometric estimations are given in Table 1. Nuclear chromatin may be stabilized by magnesium ions in magnesium chloride buffers and magnesium sulfate buffers and by spermine in the polyamine buffer [21,23,24]. In Marie's isolation buffer, the presence of glucose helps maintain nuclear integrity and prevents their clumping EDTA, a metal chelator is used to bind divalent cations, which serve as nuclease cofactors [14,23]. Sodium citrate, a mild chelating agent, and inorganic salts (KCl, NaCl) are also included in some buffers [14,26]. The pH of the solutions varies within a limited range (7.0–8.0), which is compatible with common DNA fluorochromes, and is stabilized by organic buffers (TRIS, MOPS, HEPES). Two non-ionic detergents, Triton X-100 and Tween 20, are included to facilitate nuclear release from the cytoplasm, remove cytoplasmic remnants from the surface of isolated nuclei, disperse chloroplasts and decrease a tendency of nuclei and cytoplasmic debris to aggregate.

Considering the diversity in tissue anatomy and chemistry among plant species, it is not surprising that there is no single isolation buffer which works well with all species. Further improvisations may be obtained by subtle changes in buffer composition and its pH[25,27,28]. In some difficult species, it may be necessary to increase the concentration of a detergen and isolation of nuclei from tissues rich in phenols may require addition of a reducing agent or polyvinylpyrrolidone [14,25,26,27,28,].

2.2 Sample preparation for flow cytometry

Healthy root tissue from the selected populations of *Urginea* – Gulbarga and Nagarhole were selected for flow cytometric analysis and a calibration standard was *Allium cepa*. Approximately 50 mg of young root tissue (~ 1-3cm long) from the bulbs of *Urginea*was used for sample preparation. Leaves, bulb scales, inflorescence axis and older roots (>3 cm) are not suited for flow cytometric sample preparations because of high mucilage content. Nuclei suspensions were prepared in Galbraith's buffer and the flow cytometric estimations were carried out by following the methods employed by Sayantani *et al.,* [29]. For each population, three individuals are analyzed and measured independently. The total nuclear DNA content is calculated by multiplying the known DNA content of the internal standard

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with the quotient between the 2C peak positions of the target species. Mean values and standard deviations are calculated based on the results for the three trials. For genome size estimation, approximately 50 mg of the young root tissue of *Allium cepa* was used for sample preparation following the same protocol.

2.3 Estimation of nuclear genome size

A BD FACS Calibur Flow Cytometer equipped with air-cooled argon-ion laser, 15 milliwatt, 488 nm and CellFIT software was used for analysis of samples. Cell debris and disintegrated nuclei are excluded in each run uniformly by gating the scatter profiles of the analyzed samples. For each sample 5,000 to 12,000 events (nuclei) were collected and the resulting histograms were analyzed using CellFIT software (San Jose, CA).

The 2C DNA amount of a sample is calculated based on the values of the G1 peak means:

Sample 2C DNA content = [(sample G1 peak mean) / (standard G1 peak mean)] X standard 2C DNA content (pg DNA).

The DNA amounts are reported in pg DNA. DNA in picograms can be expressed in terms of basepairs with the formula 1pg = 978Mbp. Statistical analysis was carried out by means of Student's unpaired *t*-test and one-way ANOVA test at 5% level of significance.

3. RESULTS AND DISCUSSION

The histograms obtained by subjecting the samples for flow cytometric estimations is shown in the figure 3 – 5. The genome sizes 2C content estimated by flow cytometry was revealed to be *Urginea indica* from Nagarhole being 69.73 ± 0.298 pg and *Urginea wightii* from Gulbarga being 55.21± 0.28 pg of DNA. The 1C content and the basepairs are depicted in the table 2. Variations in nuclear DNA content estimates were noted among *U. indica* plants used; however, those variations were found to be statistically significant ($P < 0.05$).

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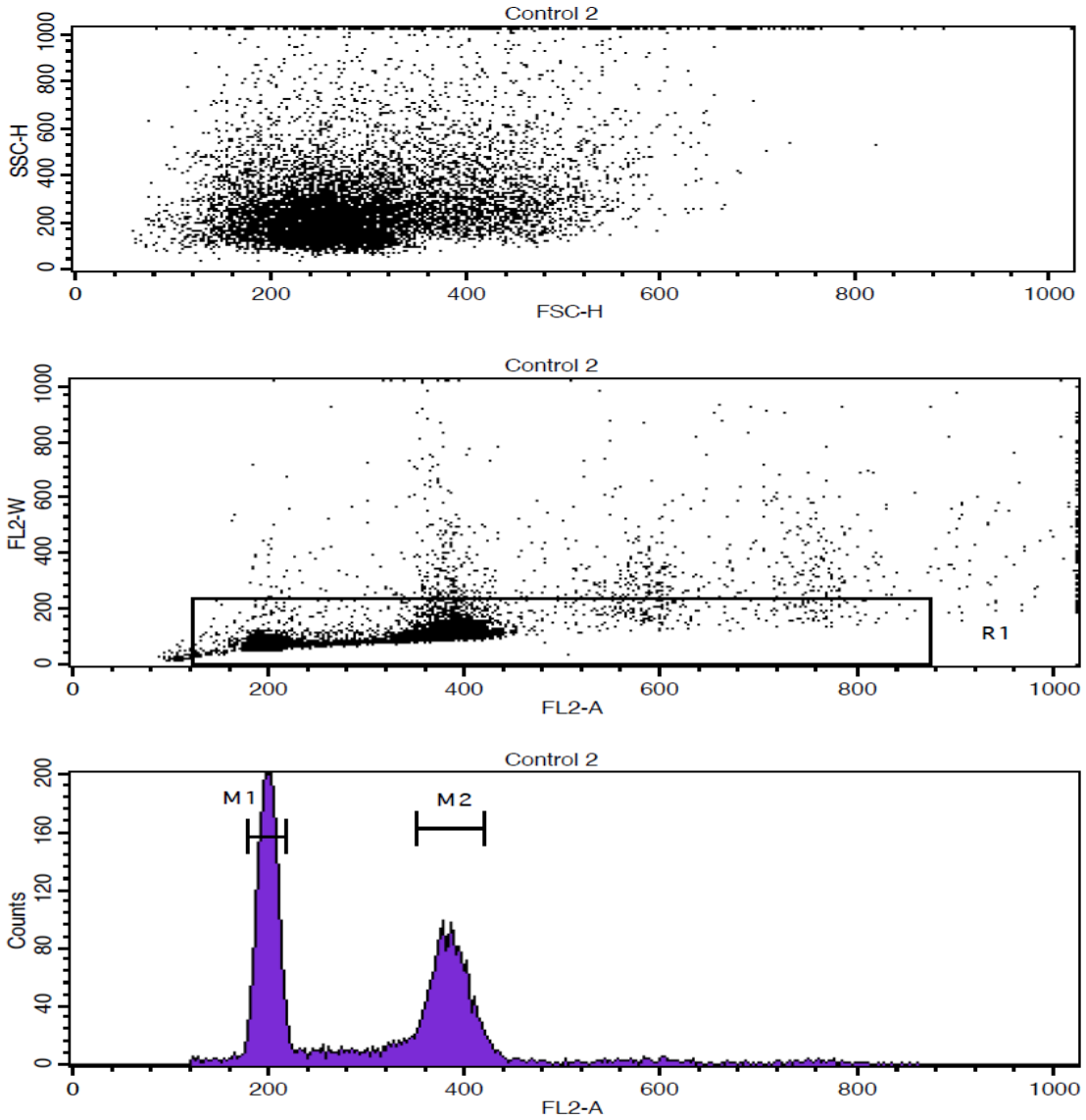

Histogram Statistics

File: Control 2
Sample ID: Control 2
Tube: Untitled
Acquisition Date: 14-Jan-16
Gated Events: 10092
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 11018

Marker	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak	Peak Ch
All	10092	100.00	91.60	292.37	274.90	36.29	251.00	207	201
M1	4650	46.08	42.20	198.71	198.52	4.37	199.00	207	201
M2	3737	37.03	33.92	383.63	383.28	4.24	383.00	98	377

Fig. 3: Propidium iodide fluorescence intensity-area (PI-A) histograms *Urginea indica* with internal standard *Allium cepa*

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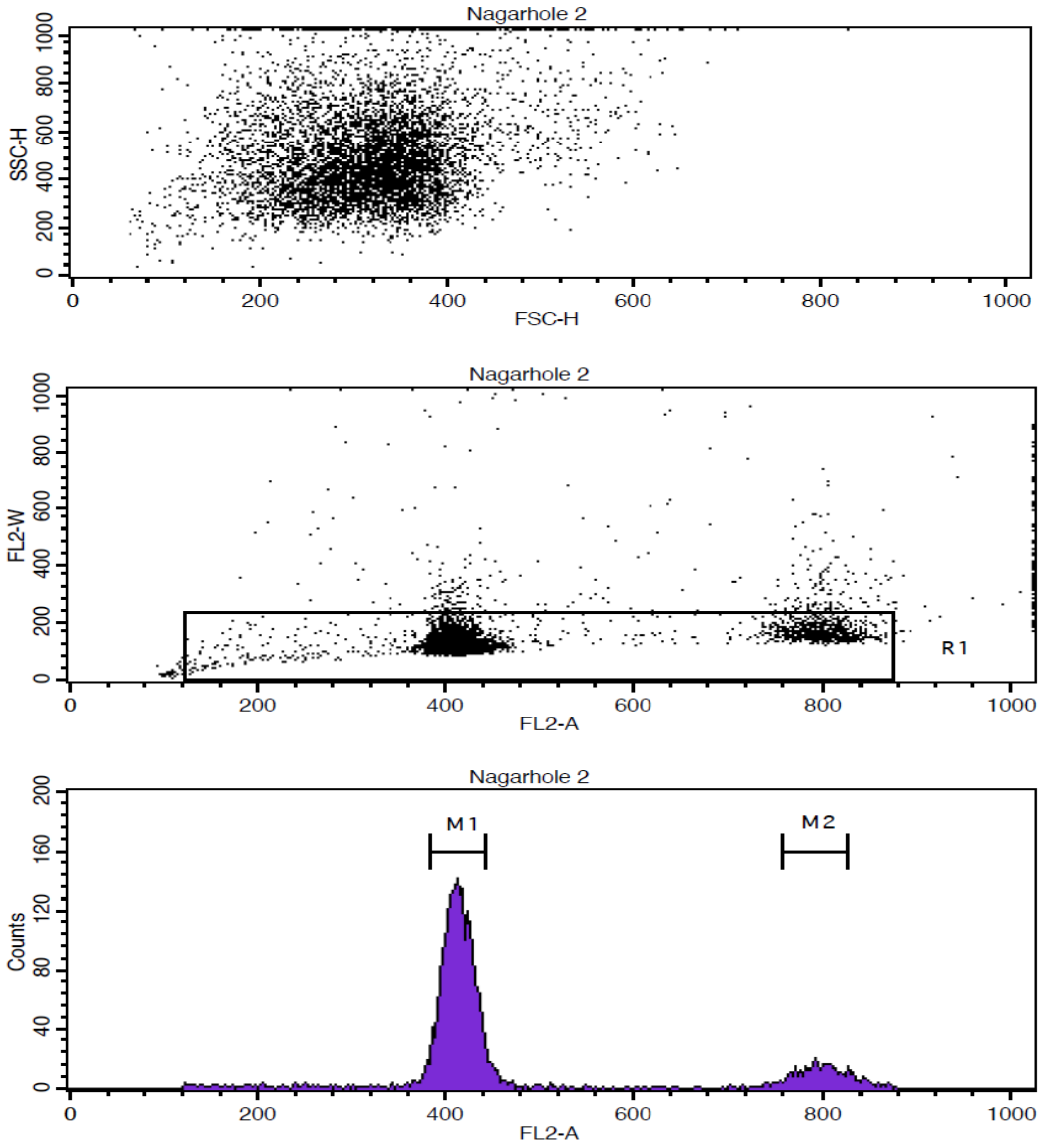

Histogram Statistics

File: Nagarhole 2
Sample ID: Nagarhole 2
Tube: Untitled
Acquisition Date: 14-Jan-16
Gated Events: 6300
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G1
Total Events: 6765

Marker	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak	Peak Ch
All	6300	100.00	93.13	460.54	443.65	30.16	414.00	141	412
M1	4867	77.25	71.94	411.49	411.26	3.29	411.00	141	412
M2	681	10.81	10.07	793.98	793.78	2.25	795.00	20	790

Fig. 4:Propidium iodide fluorescence intensity-area (PI-A) histograms *Urginea indica* – Nagarhole accessionwith internal standard *Allium cepa*

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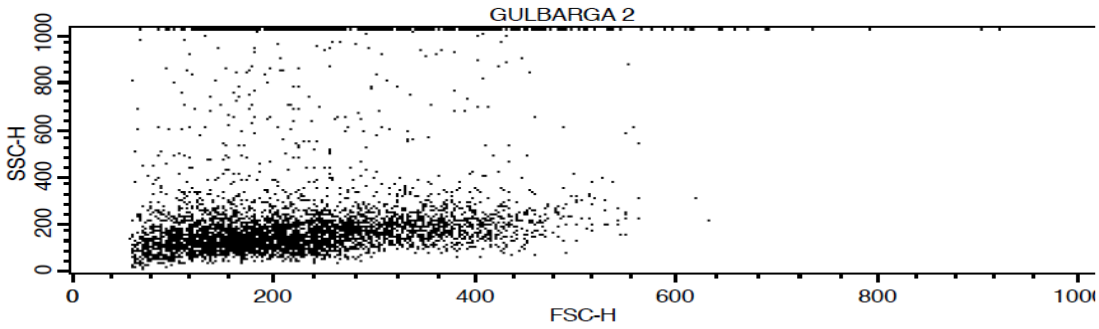

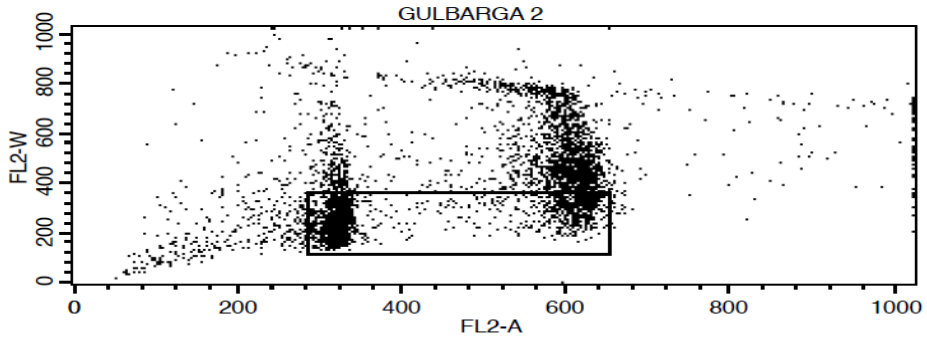

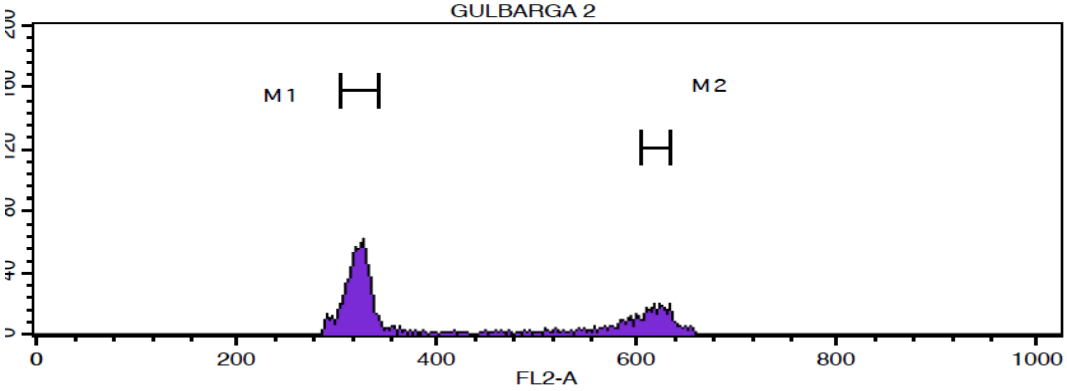

Histogram Statistics

File: GULBARGA 2
Sample ID: GULBARGA 2
Tube: Untitled
Acquisition Date: 14-Jan-16
Gated Events: 2194
X Parameter: FL2-A (Linear)

Log Data Units: Linear Values
Patient ID:
Panel: Untitled Acquisition Tube List
Gate: G2
Total Events: 5025

Marker	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak	Peak Ch
All	2194	100.00	43.66	414.50	395.58	32.12	329.00	61	327
M1	1269	57.84	25.25	322.19	322.07	2.68	322.00	61	327
M2	371	16.91	7.38	619.31	619.26	1.30	619.00	19	616

Fig. 5:Propidium iodide fluorescence intensity-area (PI-A) histograms *Urginea wightii* – Gulbarga accessionwith internal standard *Allium cepa*

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Flow cytometry is suited for analysis of populations of plants for taxonomy, population biology and ecology studies. It seems highly probable that a growing number of applications will be seen in these areas. While the estimation of relative DNA amounts for ploidy screening and some other applications usually do not represent serious problems, the use of flow cytometry for estimation of genome size is a greater challenge. C-value variation may be due to heterochromatin, polymorphism, B-chromosomes, aneuploidy and polyploidy[30]. Polyploids have the more tendency of having larger C-values compared to their diploid progenitors [31]. They have observed that mean 1C DNA content was not directly proportional to the increasing ploidy. This leads to the phenomenon of genome downsizing where DNA loss is observed in polyploids. A user-friendly powerful method for genome sizing – the flow cytometry and showed that it is fastest, works with a wide variety of materials and provides information on a very large number of nuclei. Genome size has played an important role in the evolution of plants and animals [32].

The genomes of plants have frequently been labelled as 'fluid', 'dynamic' and 'in constant flux', due in large part to the seemingly common observation of pronounced intraspecific variation in their nuclear DNA contents. In some cases, real variation within species can be explained by the differential presence of supernumerary B chromosomes but the A chromosome complement remains unchanged. However, intraspecific variation in DNA content can be attributed to recognizable polymorphisms in the A chromosomes themselves, as with heterochromatic knobs in maize or differentially deleted transposable element remnants in barley. In species of *Urginea*, the differences in genome size can be attributed to the aneuploid status of the populations, but the variations in genome size within diploids and tetraploid accessions have been reported by Shivkameshwari. Intraspecific variation in the genome size of the *Urginea* species may be attributed due to transposable elements and repetitive sequences. The polymorphic nature of *Urginea* species in their chromosome number, morphology and the adaptation to their different habitats resulted in the appearance of distinct cytotypes, morphotypes and ecotypes which led to diversification of *Urginea* and finally led to evolution. The chromosomal differences and the differences in the genome sizes should be aptly interpreted as the evidence for substantial biological diversification and therefore speciation.

Table 1: The chemical composition of the most popular nuclear isolation buffers used for flow cytometric estimations

Buffer	**Composition**	**REFERENCES**
Galbraith	45mM MgCl2 30mM Sodium citrate 20mM MOPS (3-LN-morpholino propanesul fonate) 0.1% (v/v) Triton X-100 pH 7.0	12
LB01	15mM Tris 2mM Na2EDTA 0.5mM Spermine. 4HCl 80mM KCl 20mM NaCl 0.1% (v/v) Triton X-100 pH 8.0	23
Otto	Otto I: 100mM Citric acid 0.5% (v/v) Tween 20 pH 2-3	33,34
	Otto II: 400mM Na2PO4 12 H2O pH 8-9	
Tris MgCl2	200mM Tris 4mM MgCl2. 6H2O 0.5% (v/v)	20

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	Triton X-100 pH 7.5	

Table. 2: Flow cytometric analysis of the accessions of *Urginea indica* and *Urginea wightii* studied

Sample	G_1 Mean	Avg G_1 Mean	CV	2C DNA Content (pg DNA)	1C DNA Content (pg DNA)	DNA content (in Mbp)
Control	189.71	186.56	3.72	33.5	16.75	16371.5
	184.96		3.22			
	185.02		3.2			
Nagarhole	405.33	409.55	3.39	69.80 ± 0.29	34.90	34132.2
	411.49		3.29			
	411.83		3.19			
Gulbarga	322.42	323.92	3.14	55.21 ±0.28	27.61	27002.5
	322.19		2.68			
	327.16		2.22			

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APPLICATION OF LINEAR ALGEBRA IN PEDIGREE ANALYSIS

M. Naga Maruthi Kumari[1*], Krupashree Y.N[1] and kavya H.U[1]
[1]Department of Mathematics, REVA University, Bengaluru, 560064

ABSTRACT: The present paper is focussing on the mathematical concepts from linear algebra can be applied to genetics. In order to perform such an analysis, by using several critical concepts from linear algebra, including Eigen values, Eigen vectors and diagonalization of a matrix. Throughout the study of Genetics there are many factors that take parts to study by analysing the pedigree chart for Sickle cell anemiaand it's a genetic diseases which causes the expression of defective haemoglobin irregularly shaped red blood cellsby many free radical processes. These sickle cells cause problems in the body, often blocking blood flow and causing painful attacks and sometimes stroke. Thus the report suggests, that to findgenotype distribution of the individuals affected by Sickle cell anemia for n^{th}generation considering the pedigree chart by develop a mathematical model using linear algebra concepts.

Key words: Eigen values, Eigen vectors, Diagonalization, Pedigree Analysis, Sickle cell anemia, redcells genotype distribution.

Corresponding Author : M. Naga Maruthi Kumari*PhD.
Department of Mathematics, REVA University, Bengaluru
Email Address : suma@reva.edu.in

1. INTRODUCTION

Algebra is mathematical approach of solving variables by using equations. Linear algebra takes that to another level by manipulating these variables using mathematical structures. In linear algebra use to study of vector space and linear mapping between them, that will learn how to represent the entire system of equation in the form of matrices and vectors. These eigen values and eigen vectors make understanding linear transformation very easily so that we need to have more linearly independent eigen vectors associated in a single linear transformation so,that will be useful in variety of real world problems. Here we are focussing basedon Genetics. Genetics is a branch of biology that deals with the heredity and variation of organisms. In that Sickle cell disease (SCD) is a lifelong blooddisorder characterized by red blood cells. The sickling occurs because of a mutation in the haemoglobin gene. Life expectancy of 42 and 48 years for male and females. Sickle cell disease, usually presenting in childhood.Sickle cell diseases in inheritance defective haemoglobin genes from both parents.Recently, it has been demonstrated that sickled red bloods cells are more susceptible to oxidative damage than normal red blood cells.And has a genetic component.

2. Definitions

2.1 Pedigree Analysis: A pedigree is family tree or chart which is used to trace inheritance of a trait and also it is a graphical representation of the appearance of a particular trait or diseases.

2.2 Eigen values A scalar λ is called an eigenvalue of the $n \times n$ matrix A is there is a nontrivial solution x of $Ax = \lambda x$. Such an x is called an eigenvector corresponding to the eigenvalue λ.

2.3 Eigen Vectors Eigenvectors are a special set of vectors associated with a linear system of equations (i.e., a matrix equation) that are sometimes also known as characteristic vectors, proper vectors, or latent vectors.Diagonalization for the given transition square matrix A is

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said to be diagonalizable $A = PDP^{-1}$

2.4 Sickle cell anemia: Sickle cell anemia is a genetic disorder which causes the expression of defective haemoglobin resulting irregularly shaped red blood cells, known as "sickle cells."

3. RESULT

In this paper we find genotype distribution of the individuals affected by Sickle cell anemia for n^{th}generation considering the pedigree chart by develop a mathematical model using linear algebra concepts for several generation by analysing the pedigree chart of the particular person. The following symbol used to represent the different aspects of the pedigree

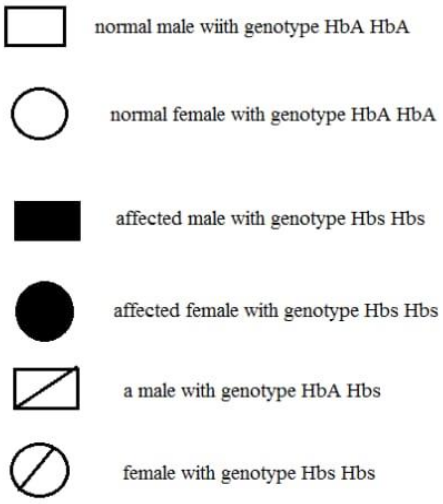

Figure :1

The following pedigree chart shows the history of Sickle cell anemiapatients in a particular family

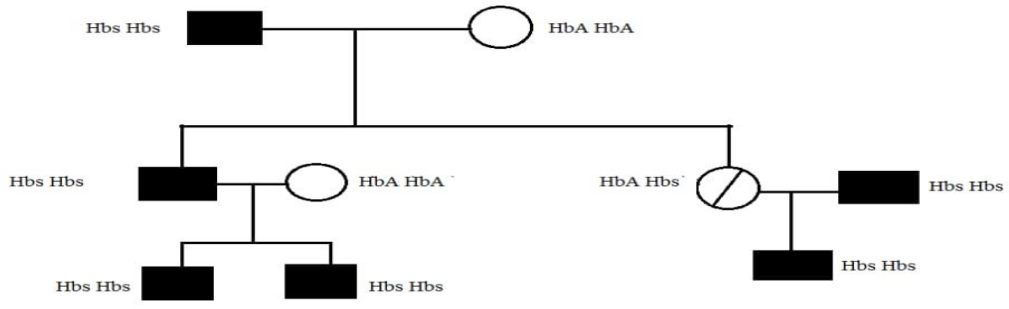

Figure :2

If the boy in 3rdgeneration marries a girl with different genotypes then the probabilities are:

3.1 Case 1: if a boy in 3rd generation marries with affected female.

Table :1

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$H_b^s H_b^s$/ $H_b^s H_b^s$	H_b^s	H_b^s
H_b^s	$H_b^s H_b^s$	$H_b^s H_b^s$
H_b^s	$H_b^s H_b^s$	$H_b^s H_b^s$

The probablilty of individuals affected by sickle cell anemia are:

Table :2

Genotype	$H_b^A H_b^A$	$H_b^A H_b^S$	$H_b^S H_b^S$
Probablity	0	0	1

Case 2:boy in 3rdgeneration marries a girl with genotypethen$H_b^A H_b^S$

Table :3

$H_b^S H_b^S$/ $H_b^A H_b^S$	H_b^S	H_b^S
H_b^A	$H_b^A H_b^S$	$H_b^A H_b^S$
H_b^S	$H_b^S H_b^S$	$H_b^S H_b^S$

The probabilities of affected individual are:

Table : 4

Genotype	$H_b^A H_b^A$	$H_b^A H_b^S$	$H_b^S H_b^S$
Probability	0	½	½

Case 3: in 3rd generation if aboy marry a girl with genotype$H_b^A H_b^A$

Table : 5

$H_b^S H_b^S$/$H_b^A H_b^A$	H_b^S	H_b^S
H_b^A	$H_b^A H_b^S$	$H_b^A H_b^S$
H_b^A	$H_b^A H_b^S$	$H_b^A H_b^S$

the probabilities of affected individual

Table :6

Genotype	$H_b^A H_b^A$	$H_b^A H_b^S$	$H_b^S H_b^S$
Probability	0	1	0

The initial matrix is

$$X0 = \begin{bmatrix} 1/3 \\ 1/3 \\ 1/3 \end{bmatrix}$$

The transition matrix obtained:

$$A = \begin{bmatrix} 0 & 0 & 1 \\ 0 & 1/2 & 1/2 \\ 0 & 1 & 0 \end{bmatrix}$$

The Eigen values for a matrix A are ½, 0,0

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The Eigen vectors are :

$$V1 = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}$$

$$V2 = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}$$

$$V3 = \begin{bmatrix} 0 \\ 0 \\ 0 \end{bmatrix}$$

Therefore the offspring's are not affected by diabetes.

4. CONCLUSION

The study suggested that sickle cell anemia is an inherited blood condition which is common among the people,The sickling occurs because of a mutation in the haemoglobin gene. Life expectancy of 42 and 48 years for male and females. Sickle cell diseases in inheritance defective hemoglobin genes from both parents. In this paper the pedigree chart shows the family history of an individuals affected by sickle cell anemia where the result in this study shows that the genotype distribution of the population affected by sickle cell anemia is zerofor n^{th}generation developedby using a mathematical modelincluding eigen value, eigen vectors, diagonalization.Diagonalization is the easiest method where we can find the solution very easily.The obtained result shows that are not affected.

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WIDESPREAD OCCURRENCE OF ENDOSYMBIONT *WOLBACHIA* AND PHAGE WO INFECTION IN *DROSOPHILA* SPECIES

N. M. Guruprasad[1] * and H. P. Puttaraju[2]
[1]Department of Biotechnology, School of Applied Sciences, REVA University
Bangalore-560064,
[2]Department of Biological Sciences, Bangalore University,Bangalore-560056

ABSTRACT: Intracellular endosymbiont *Wolbachia* are maternally inherited cytoplasmic alpha-proteobacterium found in insects and other invertebrates. The evolutionary success of *Wolbachia* is due in part to an ability to manipulate host reproduction. A array of reproductive manipulations induced by *Wolbachia* in its host are Cytoplasmic Incompatibility (CI), feminization of genetic males, male killing, parthenogenesis and speciation. From the previous surveys 16-76% of arthropod species infected with *Wolbachia*. Two major phylogenetic subdivisions (A and B) of *Wolbachia* occur. In the present study, using Polymerase Chain Reaction (PCR) assay for A and B group *Wolbachia* by using ftsZ A and ftsZ B group primers in 11 *Drosophilla* species, we found that 100% *Wolbachia* double strain infection. Apart from *Wolbachia* infection, we also screened for Phage WO infection by using phage WO specific primers or2 and orf7. The phage WO specific amplification were positive for Phage WO infection in all tested *Drosophila* species. These results indicate widespread infection of both *Wolbachia* and Phage WO in *Drosophila* species and provide a affluence information relevance of these infection patterns to the evolution and dynamics of *Wolbachia*/phage/host symbiosis and potential uses of *Wolbachia* virulent strains of *Drosophila* to over come the menace of vectors of human and agriculture pests.

Key Words: *Wolbachia*, Phage WO, Infection and *Drosophila*.

*Corresponding author: N. M. Guruprasad
Department of Biotechnology, School of Applied Sciences, REVA University
Bangalore-560064, Email- guruprasad.nm@reva.edu.in

1. INTRODUCTION

Wolbachia are maternally inherited obligatory intracellular symbionts, which infect a wide range of arthropods and filarial nematodes [1].It is a member of the order Rickettsiales and is closely related to the insect vectored mammalian pathogens Anaplasma and Ehrlichia. Ten supergroups of *Wolbachia* have been identified within the species *W. pipientis* [2]. Super groups A and B are common in insect symbionts which probably diverged from one another 50-60 MYA[3]. *Wolbachia* are known to induce various reproductive alterations in their hosts, including Cytoplasmic Incompatibility (CI), thelytokous parthenogenesis, feminization and male killing [4,5,6]. At least 16% to 76 % of neotropic insects are infected with *Wolbachia*[7,8,9] .In addition, some strains do not induce any apparent reproductive effect. Surveys have also shown that, in addition to insects, *Wolbachia* are common in mites[10], terrestrial isopods [11] and filarial nematodes biodiversity [12]. Groups A and B contain the *Wolbachia* variants infecting the insect, mite, and crustacean [3,13], whereas groups C and D

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harbor the filarial nematode *Wolbachia* [12]. Recently, group E has been reported from the springtail *Folsomia candida*[14] and group F from termites[15].

The presence of phage WO in *Wolbachia* was first suspected when Wright *et al*. (1978) observed phage –like particles in *Wolbachia* from the mosquito *Culex pipiens*[16]. Recently, Masui *et al*. (2000, 2001) characterized the phage WO and showed that it can be either lysogenic and integrated into the *Wolbachia* chromosome, or lytic and free in the cytoplasm [17, 18]. Because phage WO often bring to their bacterial host important functions involved in virulence (Such as the cholera toxin of *Vibrio cholerae*, [19] or antibiotic resistance (as *Pseudomonas aeruginosa*, [20], phage WO appears to be realistic candidate to explain the diversity of effects that *Wolbachia* induces in insects. The lytic activity of the phage WO is well documented [21,22], making its prolonged persistence in *Wolbachia* strains puzzling observation. Indeed, strong selection pressures acting on endosymbiotic prokaryotes tend to eliminate parasitic DNA such as repeated DNA or phages [23]. Phage WO is one of the rare reported cases of bacteriophge infection in an intracellular bacterium [24], suggesting that phage WO might provide some factors of importance to *Wolbachia*, for example by contributing to the reproductive alterations they induce in their hosts.

The drosophila is used as a model organism in genetic and evolutionary studies. This made us to know the infection status of *Wolbachia* and its Phage WO infection in 11 *Drosophila* species in present study was screened for *Wolbachia* A and B strain and Phage WO by using specific primers.

2. MATERIALS AND METHODS

2.1 Collection of *Drosophila* species

Drosophila sp. was collected from the Drosophila Stock Center, Department of Zoology, University of Mysore, India. The collected specimens were brought to the laboratory and stored in the -40^{o} C until the isolation of genomic DNA.

2.2 Isolation of genomic DNA and PCR diagnosis for *Wolbachia* and phage WO infection

The genomic DNA of *Drosophila* species were extracted by proteinase – K and SDS lysis method as mentioned in Sambrook *et al.* (1989)[25]. The genomic DNA was resuspended in 50 µl of TE (10 mM Tris-HCl. 1 mM EDTA , P^{H} 8.0).The Polymerase Chain Reaction (PCR) assay was carried out based on specific amplification of the *Wolbachia* primers ftsZ Adf – 5^{1} CTC AAG CAC TAG AAA AGT CG 3^{1} ftsZ Adr – 5^{1} TTA GCT CCT TCG CTT ACC TG 3^{1} and ftsZ Bf – 5^{1} CCG ATG CTC AAG CGT TAG AG 3^{1} ftsZ Br – 5^{1} CCA CTT AAC TCT TTC GTT TG 3^{1}super group (*Wolbachia* cell division protein) and phage WO specific amplification primers **or f7** WOF – 5^{1} CCC ACA TGA GCC AAT GAC GTC TG -3^{1} WOR - 5^{1} CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC 3^{1} andORF2F- 5^{1} GCAGGGCTATATTTTGGCGAGAA 3^{1} ORF2R- 5^{1} AACTCCATTAAAACTTCCCTGGC 3^{1} and phage WO open reading frame (orf), which was synthesized in Sigma Aldrich, India.

The PCR was carried out with PTC 200 of MJ Research Thermocycler, in 25µl reaction mixture containing 2.5µl of 10X PCR buffer, 0.5µl of dNTP's (10mM each), 2.5 µl of 2.5mM $MgCl_2$ and 0.5 U Taq DNA polymerase (New England Biolabs, England), 1µl of both forward and reverse primer (5 pmol), 20 ng of template DNA. The final volume was adjusted to 25µl by milique water. The PCR was carried out with a cyclic condition of initial denaturation at 94^{o}C for 5 min followed by 35 cycles with denaturation at 92^{o}C for 1 min,

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extension 72° C for 1 min, final extension at 72° C for 10 min at specific hybridization temperature. The presence of amplified PCR products was checked by electrophoresis on 1.5% agarose gel running in 1X TBE (89.2mM Tris HCl, 88.9mM Boric acid and 2mM disodium EDTA) buffer for a length of about 5 cm with a constant voltage of 70V. The gel was stained with 0.5 µg/ml ethidium bromide prior to casting. Gel documentation was done by using Alpha digi doc documentation system.

3. RESULTS AND DISCUSSION

3.1 *Wolbachia* infection in *Drosophila* species:

Wolbachia specific sequence amplification preferred for characterizing *Wolbachia* strains. We amplified fragments of ftsZ A and ftsZ B *Wolbachia* cell division protein genes 11 individuals from each of the *Drosophila* species groups tested positive for *Wolbachia*. The PCR amplification for the presence of *Wolbachia* super group A and B yield positive results showing that *D. melonogaster, D. ananassae, D. nasuta, D. neonasuta, D. sulfurigoster, D. albomicans, D malerkolliana, D. photicella straita, D. nigrer, D. bipectinata* and*D. simulans* are infected with both the strains of *Wolbachia* A and B group. Fig- 1,2 .Table-1.In all cases, the results were consistent with both sets of ftsZ A and ftsZ B of primers. Positive amplification of *Wolbachia* A and B group were obtained from all samples of *Drosophillasp.* consistently.

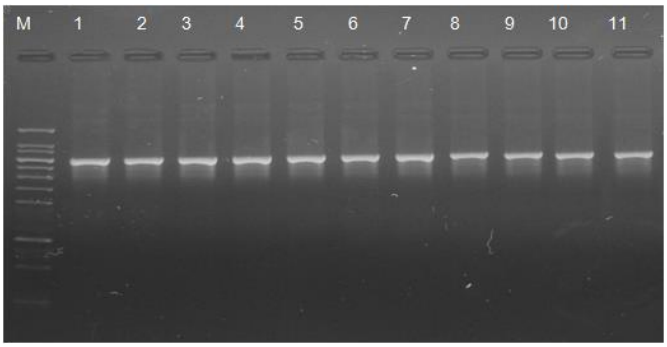

Figure 1. Gel photo showing *Wolbachia* strain A amplification in *Drosophila* sp. M is Marker 1-11 is different species of *Drosophila*.

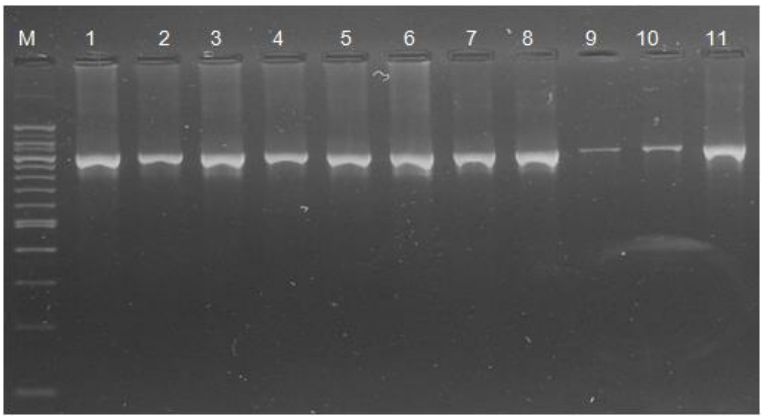

Figure 2. Gel photo showing *Wolbachia* strain B amplification in *Drosophila* sp. M is Marker 1-11 is different species of *Drosophila*.

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Further it is also found that individual *Drosophila* species were uniformly infected with both A and B *Wolbachia* strains. Based on the present result it can be predicted that *Wolbachia* is widespread in the *Drosophila* species used for the present study. We conclude from these results that the bacteria infecting the *Drosophila* species with the infection of both strains of *Wolbachia* A and B.

In *Drosophila*, *Wolbachia* is exclusively maternally transmitted [26]. Thus, the spread of one *Wolbachia* genotype, either by CI or by fitness benefits to the host will affect the distribution of mitochondrial DNA (mtDNA) haplotypes in the host species. The infection status of individuals in natural populations in combination with their mtDNA haplotype could provide more insight into the population dynamics of the *Wolbachia* infection. In this study, we analyzed *Drosophila* species for *Wolbachia* infection and found 100% *Wolbachia* infection in the present tested populations.

Wolbachia were probably first described in *D. melanogaster*- by Wolstenholme(1965)[27], who described bacteriod particles in salivary glands and ovaries of two infected lines with reduced numbers of offspring of infected *vs.* uninfected males, consistent with CI.During the past two decades, *Wolbachia* infections and *Wolbachia* induced CI have been reported from several *Drosophila sp*. *Wolbachia* infection in *D. melanogaster* population is associated with weak incompatibility in the laboratory[22]. This infection is widespread among the populations around the world. In Australia, most populations are polymorphic for the infection but can differ markedly in infection frequency. Because the infection causes only weak laboratory CI, its persistence is difficult to explain if there is incomplete maternal transmission[23] .

Table-1 List of *Drosophila* species used in the present study

Sl. No.	Species Name	*Wolbachia*		Phage WO		Phenotype induced [22]
		ftsZ-A	ftsZ-B	Orf-2	Orf-7	
1	*Drosophil melanogaster*	+	+	+	+	CI
2	*Drosophila ananassae*	+	+	+	+	CI
3	*Drosophila nasuta*	+	+	+	+	CI
4	*Drosophila neonasuta*	+	+	+	+	CI
5	*Drosophila sulfurigoster*	+	+	+	+	CI
6	*Drosophila albomicans*	+	+	+	+	CI
7	*Drosophila malerkolliana*	+	+	+	+	CI
8	*Drosophila photicella straita*	+	+	+	+	CI
9	*Drosophila nigrer*	+	+	+	+	CI
10	*Drosophila bipectinata*	+	+	+	+	CI
11	*Drosophilla simulans*	+	+	+	+	CI

3.2 Phage WO infection

Results obtained from the phage WO specific PCR assay revealed that there is presence of Phage WO in all the tested populations of *Drosophila sp*. The presence of Phage WO was detected by using phage WO specific primers orf-2 and orf-7, a gene coding for a minor capsid protein of page WO. PCR specificity on phage WO infection in tested all *Drosophila* individuals was detected the Phage WO infection in all *Wolbachia* infected

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Drosophilla species. Fig-3,4. Table-1.

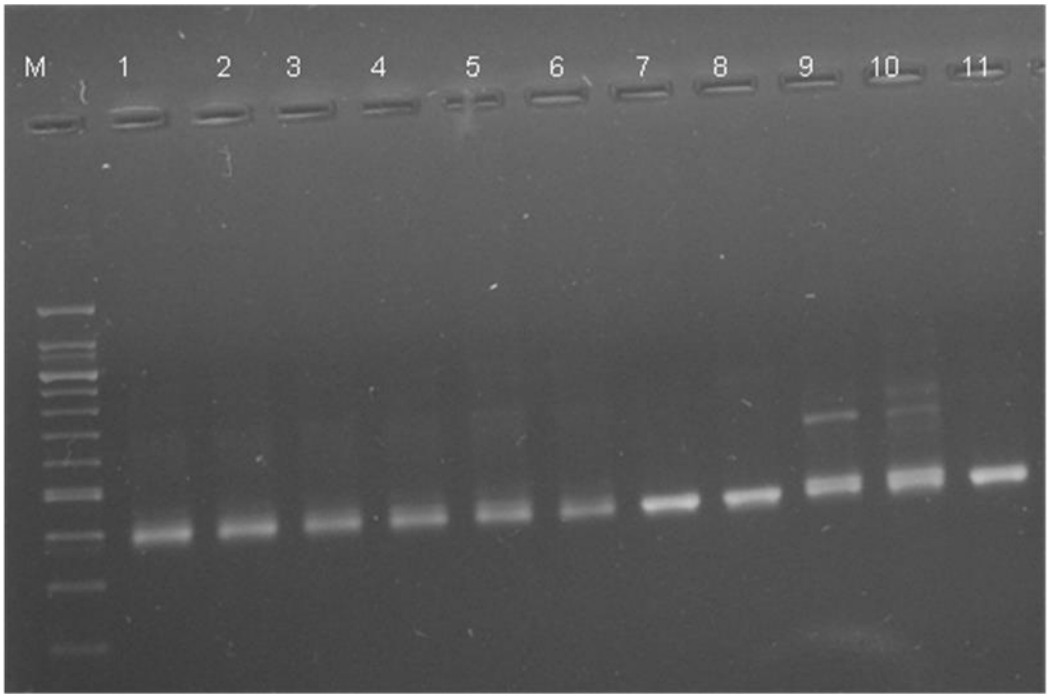

Figure 3. Gel photo showing Phage WO by using ORF2 primer amplification in *Drosophila* sp. M is Marker 1-11 is different species of *Drosophila*.

Figure-4 Gel photo showing Phage WO by using ORF7 primer amplification in *Drosophila* sp. M is Marker 1-11 is different species of *Drosophila*.

Several recent studies revealed that phage WO is widespread in *Wolbachia* genomes [21] and the detection of particles has lent support to the idea that phage WO genes are activated and mobile. This notion was strengthened by the lack of congruence between *Wolbachia* and phageWO phylogenies established with the orf7 sequence suggesting horizontal phage exchange between bacteria co-infecting the same intracellular environment.

However, the discoveries of phage WO in two unrelated bacterial endosymbiont systems, the c-Proteobacteria secondary symbiont of aphids [20, 25] and the widespread a-Proteobacteria arthropods, *Wolbachia,* have raised new questions about whether bacteriophages can play important roles in the evolution of some endosymbiont genomes. Determining whether these phage WO are active and important contributors to genomic diversification in their endosymbiont hosts remains an important area of research for these elements. Studies of phage WO gene expression and particle enrichment support the activity and lytic ability of these phages. For endosymbiotic bacteria that have an intracellular lifestyle and, therefore, reduced opportunities for genetic exchange, active bacteriophages could constitute both a serious threat and central source of evolutionary innovation, even more so than in free-living bacteria [26-28].

4. CONCLUSION

The present study clearly reveals the infection of both the strains of *Wolbachia* A and B group in all tested *Drosophila* species. Further, the infection of Phage WO infection in *Wolbachia* infected *Drosophila* species clearly indicated that a triplicate association of host, *Wolbachia* and Phage WO.The association between host, *Wolbachia* and phage WO appears complex

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and raises novel issues on the dynamics of *Wolbachia* and phage WO infection in *Drosophila species*. The study will have a broader implications to exploit the possible role of *Drosophila* virulent strain of *Wolbachia* and its phage WO for novel based technology to combat arboviruses and its vectors in particular health and agricultural insect pests.

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BIO-HYDROGEN: AN ALTERNATIVE AS A CONVENTIONAL APPROACH TO FOSSILS

Ankit Kumar Dubey, Ligi Milesh*, Giram Pranoti Datta
[1]Department of Biotechnology, School of Applied Sciences, REVA University,Yelahanka, Bangalore -560064

ABSTRACT: The greatest concern for the world today is the environmental pollutions that is mainly taking place due to the rapid utilization of natural resources and industrialization. The most common root for this is the release of harmful gases by the combustion of fossil fuels, which causes various environmental issues. There is need to overcome these issues by developing advance and alternate energy sources, which can mimic this issues without any impact in the surroundings. Although the conventional fuels are depleting at a higher rate today like reserves of oil, coal and natural gas, therefore research is carried out for the sustainable environment for future generations. However, hydrogen assures to be a potential clean, renewable and environmental friendly energy source because of its easy conversion accessibility to electricity by fuel cells and it does not involve any emission of greenhouse gases like CO2, which are released from the combustion as a clean fuel. Biological hydrogen production generally can be carried out by two mechanisms—the fermentation and photo biological production. Photo biological hydrogen production has the advantage that utilizes solar radiation to run the operation but progressive reactor designs are required to achieve moderate solar radiation conversion efficiencies and H2 production rates. The process of fermentation utilizes free carbon as source of energy in agricultural by-products or wastes. However, the feasibility of fermentative hydrogen production depends upon the choice of the substrate.

KEYWORDS: Hydrogen gas, environment, solar radiation, biophotolysis, fermentation.

***Corresponding author:** Dr. Ligi Milesh, PhD, Assistant Professor,
Department of Biotechnology, School of Applied Sciences,
REVA University**,** Bangalore, India
Email id: ligimilesh81@gmail.com

1. INTRODUCTION

The greatest concern of the global population in today's world is the environmental pollution.This is mainly taking place due to the rapid growth of population and their demands, giving rise to rapid utilization of resources and the industrialization.The rate of usage of these non-renewable resources is resulting in the shortage of the conventional resources such as reserves of coal, petroleum and natural gas [1]. The increase in the utilisation of these non-renewable resources has led to the release of large amount of CO2 and other harmful gases in the environment, which is the noble cause of various environmental pollutions[2]. This is not only leading inpolluting our world even further, that but also helps in the broadening of the earth ozone layer that protects our earth from UV rays[3].Therefore in order to reduce the inducement of these problems there is a need for the development of eco-friendly and cleaner form of energy, which can mimic the usage of these resources and reduce the level of pollution in the environment. Therefore, researches are being conducted to provide a sustainable environment for future generations.

2. BIOHYDROGEN

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The bio-hydrogen gas is the production of gas as an alternative source of energy for the usage in our day-to-day life activities as a fuel from the biological sources such as the feedstocks like bioenergy crops and organic waste from processing of wood and agricultural wastes[1]. The matter used for the generation process are the renewable sources of energy by the action of microbes. This technology has the potential to replace the fossil source of energy, which release large amount of greenhouse gases, thus sustaining the power supply of the global world.

2.1. Why biohydrogen as a fuel?

Hydrogen is considered the environmental friendly, potentially clean and a renewable source of energy. Since 90% of the hydrogen gas are produced from the fossil fuels [4]the production strategies developed for H2 production from biological feedstocks is a promising technology towards the conservation of the fossil fuels and generating a fuel with low density and high heating value, which surely be a potential fuel in the upcoming future[5].Since the density of hydrogen and boiling point is quite low, there becomes a trouble in storing it but it is still easier than storing of the electrical energy[6]. The table shows the comparison of heat of combustion low heating value for different fuels.

Fuel	Heat of combustion low heating value, KJ/g	Relative amount of fuel per unit volume required to equal H_2 heat *content by weight	Boiling point, °C	Storage
Hydrogen H_2	119.66	1	-253	Very difficult
Methane	50.208	2.4	-162	Difficult
Methanol	20.08	6	65	Medium
Ethanol	26.78	4.4	78	Easy
Gasoline	44.35	2.7	125	Easy

Table 1: A comparison of a few chosen fuels [6]

2.2. Feed stock sources for biohydrogen production

The sources of feedstocks for the production of bio hydrogen varies from process to process and the technology employed. The parameters that define the substrates as feedstock for the production of Biohydrogen depends on the factors like availability, cost of production, carbohydrate content, biodegradability and the storage process**.**Because of the simplicity and the higher rate of biodegradation, simple sugars are generally preferred for this process like glucose, lactose and sucrose[7].The feedstocks can be classified as sources of biomass dependent on food crops, sludge waste, food and agricultural wastes, industrial wastewater, algae and carbohydrates [8]**.** However, there are other sources of materials for feedstocks for H2 production, which includes lignocellulose materials (having high content of lignin like wood, straw, grasses etc.)[9]Sucrose based materials (sugarcane and molasses), Starch based materials (wheat, corn, sweet potato, sorghum), Livestock: Bovine manure[10-11], Sewage[12]. Moreover nowadays microalgae is gaining proficient as a major source of feedstock for biofuel production due to its high greenhouse capturing ability, rapid cultivation and growth rate, and short harvesting period[13]

3. PRODUCTION PROCESSES

Biological hydrogen production generally can be carried out by two main mechanisms, which are classified based on use of light energy.

3.1. Photo biological production (bio photolysis): This method is of two types based on the lysis of water molecule in one-step or two steps.

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3.1.1 Direct bio photolysis:

The photo biological process(bio photolysis) in which the photosynthetic production of biohydrogen is achieved by spitting of water moleculeinto the hydrogen and oxygen in presence of light energy (chemical reaction sowed below) during the light reaction of the photosynthesis occurring in the thylakoid membrane of the granum.

$$2H2O + \text{Light energy} \rightarrow 2H2 + O2$$

The PSI AND PSII absorb the light and boost the electron at high energy level. This electrons reduces the all other proteins and complexes present in the thylakoid membrane in cascading manner of the non-cyclic and cyclic photophosphorylation and finally replaced with the electron from the water molecule to stabilize the chloroplast of both the photosystem [14]. The splitting of water molecule release oxygen that we use for breathing while the 2H+ ion and electrons are generated [15]. The H+ ions are used to transfer electrons from the thylakoid lumen to the stroma of the chloroplast through the ATP synthase to generate ATPs in all the green plants but in case of the green algae and cyanobacteria the enzyme hydrogenase uses this 2H+ ions and 2 electrons transferred from PSI via PSII to ferredoxin (Fd)for the production of hydrogen [16]. The green plant cannot produce hydrogen due to lack of hydrogenase enzyme even photosystem is active (in presence of the sunlight). The overall process in general is represented in the following diagram.

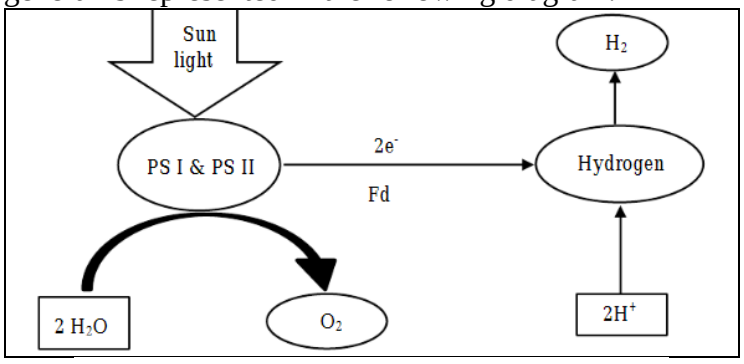

Fig 1: Schematics of direct biophotolysis [14].

3.1.2 Indirect bio photolysis:

The hydrogen production by indirect bio photolysis occurs in two steps: Photosynthesis and Fermentation. The product of the photosynthesis i.e. carbohydrates accumulate and utilised as a substrate in fermentation to produce the hydrogenas shown in following reaction [16].

$$12H2O+6CO2+ \text{Light energy} \rightarrow C6H12O6 + 6O2 \text{ (Photosynthesis)}$$

$$C6H12O6 + 12H2O + \text{Light energy} \rightarrow 12H2 + 6CO2 \text{ (Fermentation)}$$

The mutant strains of *A. Variabilis* are developed which has potential of hydrogen production with the rate of the order of 0.355 mmol/h per liter by indirect bio photolysis[14][17].

3.2. Fermentation: The production of hydrogen in absence of the oxygen (anoxygenic) is divided into two types based on the presence and absence of the light energy.

3.2.1 Photo fermentation

This process of production of hydrogen is same as the direct bio photolysis except some differences:

- The photosynthetic organisms produces the hydrogen by this method lacks Photosystem II (PSII) and perform the photosynthesis with Photosystem I (PSI) for hydrogen production.
- No oxygen is involved in this process (anoxygenic photosynthesis)
- Not only glucose other carbohydrates and organic acids can used as a substrate. The need of light energy is common requirement in both the methods).

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$C_6H_{12}O_6 + 12H_2O + \text{Light energy} \rightarrow 12H_2 + 6CO_2$ (Carbohydrate as substrate)

$CH_3COOH + 2H_2O + \text{Light energy} \rightarrow 4H_2 + 2CO_2$ (Organic acid as a substrate)

3.2.2 Dark fermentation

The hydrogen is produced in the absence of both oxygen and light energy, which is the main advantage to make this process useful for the commercialization and use at a large scale.Recently in 2018, the scientists of CSIR-IICT Hyderabad also used this technique(Acidogenic fermentation) to produce the hydrogen successfully at large scale using the vegetable waste as a substrate.*Clostridium* [4][18][19],*Enterobacter* [5][15][20]and *Escherichia* [21-22]all these large group of anaerobic bacteria are studied for their ability to produce the hydrogen.

Theoretically maximum of 4 mol H_2 is produced from 1 mol of glucose with acetic acid as the end-product, 2 mol H_2 is produced from 1 mol of glucose with butyrate as the end-product [23].The amount of hydrogen production by dark fermentation highly depends on the pH value, hydraulic retention time (HRT), gas partial pressure and Flocculation. There is need to produce the anaerobic mutants strains which will have high yield/efficiency to produce hydrogen and make it automated for the production of hydrogen in large scale in near future.

4. ENVIRONMENTAL IMPACT

Hydrogen is one of the promising approach towards the pollution free fuel in the environment that causes minimal pollution effect or no effect at all to the living organisms in the surroundings in the environment.The burning of hydrogen releases no harmful chemicals in the environment as that of the fossil fuels which releases large amount of gases like oxides of C, N, S etc. [24]. This approach is the basisof hydrogen as a fuel in the environment conferring advantages over fossil fuels. The production of hydrogen gas as a fuel is only clean with the attribution of technologies required to produce it. If the production strategies involve produces H_2 gas without any emission of greenhouse gases it will truly form the basis of a sustainable form of energy. Thus,the usage of this technology as a fuel could eventually help us to eliminate greenhouse gases from the surroundings, which leads to environmental problems[25].

5. CONCLUSION

In the conclusion, the raw materials compulsory for the hydrogen production are facing shortage of resources due to the intensive needs and demand of the humankind for their sustainability. Thehydrogen holdsan approach for the clean and better fuel taking into consideration the usage of renewable resources, which will preserve the non-renewable resources and the environment from pollution hazards. The process of production of hydrogen from biological sources makes the use of bio-feedstocks that can be anaerobically fermented(dark fermentation) and biophotolysed to release H_2 without any harmful release of chemicals in the surroundings. This will minimise the usage of fossil source of energy, thus sustaining the aesthetic value of the environment and the power supply of the global world.

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REVIEW ON INHIBITORS FOR PROTEASE AND POLYMERASE PROTEIN OF HEPATITIS C VIRUS (HCV)

Pooja R Karkera[1*], Abhishek Jha[1], Anjali Singh[1]
[1]Department of Biochemistry, REVA University, Bangalore.

ABSTRACT: Hepatitis infection can often lead to cirrhosis of the liver. Around 150 million people around the world are permanent careers of Hepatitis C. The impact of Hepatitis C in being the leading cause of cirrhosis and hepatocellular carcinoma has led to a lot of study on studying the steps and life cycle of the virus, interaction with host factor and pathway to host and HCV interaction. Development of mainly crystallisation to establish potent antiretroviral therapies has lead to approval of replication of antiviral intervention. Mainly two viral enzymes that targets for antiviral therapy are Protease NS3-4A and the RNA dependent RNA polymerase NS5B. New anti-HCV agents have been developed based on interferon and ribavirin based therapies. There are two protease inhibitors, telaprevir (VX-950, Vertex) and boceprevir (SCH 503034, Schering-Plough); and three polymerase inhibitors, valopicitabine (NM283, Idenix), R1626 (Roche), and HCV-796 (Viropharma). Current scenario, HCV NS3/4 protein (protease), NS5B (polymerase) and NS5A protein is focused by direct antiviral agents (DAA) targets. First generation drugs such as boceprevir and telaprevir which are protease inhibitors shows cure rate increases by 40%-70% for genotype -1 patients. Whereas, the second generation DAA includes Asunaprevir (sunvepra), Sofobuvir (sovaldi), Simeprevir (olysior) has ability to increase cure rate by 90% without need of interferons and effective treat all HCV genotype. As the drugs have its own limitations, nowadays research is mainly focused on plant extract and phytochemical which has potential for anti-viral activities towards virus. Such as Naringenin which are the viral assembly and releases inhibitors used for HCV from. There is also some bioactive compound such as flavanoids, triterpenes, ligans etc which can inhibit some essential viral enzymes such as HCV protease.

Keywords: Hepatitis C virus (HCV), protease and polymerase, cirrhosis, HCV inhibitors, interferon, interferon-free, natural products, drugs *Telaprevir, Boceprevir,Asunaprevir,Simeprevir* and *Sofosbuvir.*

***Corresponding Author**: Pooja R Karkera
Assistant Professor, School of Biochemistry, Reva University, Bangalore, India.
Email address: poojarkarkera@reva.edu.in

1. INTRODUCTION

HCV belongs to the family *Flavivirdae* comprising of 9.6kb positive single stranded RNA genome. HCV signifies to be a global dilemma which spectacle the variability of genome translated into 6 genotype and more than 80 subunits [1]. The genome of HCV has 5' untranslated region (UTR) which works as an internal ribosomal entry site (IRES). Hepatitis C virus infection affects approximately 170 million people worldwide**.** Hepatitis C virus (HCV) is a global health concern responsible for most of liver diseases [2]. HCV causes acute and chronic infections where acute infection is a non life threatening disease and chronic is a life threatening disease. About 15%-45% develops acute infection and the remaining 55%-85% develops chronic infection [1].

2. LIFE CYCLE OF HEPATITIS C VIRUS (HCV)

The life cycle of hepatitis C virus (HCV) is linked to the lipid metabolism of hepatocyte

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where the HCV genome interacts with core protein to form the nucleocapsid that is surrounded by a lipid membrane called viral envelope. The HCV particle shows an unusual low buoyant density and is associated with lipoproteins [1]. ApoE is associated with infectious HCV particles which thereby influences HCV assembly and entry. HCV envelop glycoproteins are the viral determinants of HCV entry into hepatocytes. The entry of HCV into hepatocytes is highly complex process which involves a series of host cell factors such as, SRB1, SD81, CLDN1, and OCLN which acts as entry factors. HCV replication takes place in double membrane vesicles which induces rearrangements of intra cellular membranes to create a membranous web in the cytoplasm. HCV hires many host cell factors to replicate its genome. HCV assembly takes place at the proximity of cytosolic lipid droplets with the help of cellular factors. Hence, HCV assembly is tightly connected to the biogenesis of VLDL thereby, causing liver cirrhosis [2].

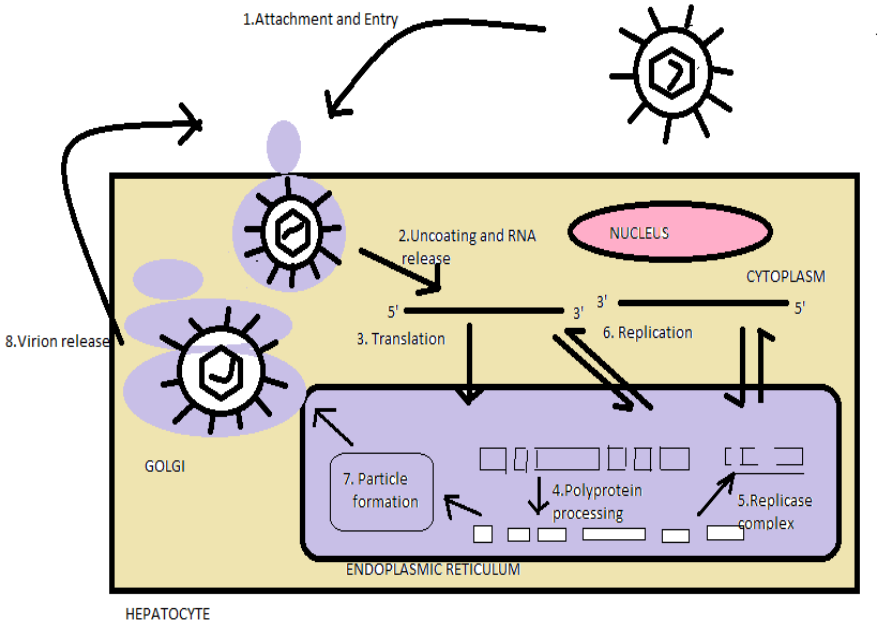

Fig 1: Hepatitis C Virus life cycle

3. PATHOGENESIS OF HCV

HCV RNA is composed of 9400 nucleotides constituting 5' and 3' as a non coding regions. Initiation of translation process begins from 5'non-coding region and produces 3000 amino acids at internal ribosomal entry site (IRES) [3]. These polypeptides undergoes modification after translation by various protease enzymes produced by host and virus and leads to the formation of:

a. Three structural proteins: HCV core forms nucleocapsid envelop of glycoproteins E1 and E2 which plays vital role in virus entry into host.

b. And seven other non-structural proteins are: NS1/p7 which codes for an ion channel, Cysteine protease formed by NS2, Serine protease and RNA helicase is coded by NS3, NS4 forms a co-factors for serine protease, NS4B helps to alter the membrane, phosphoproteins are formed by NS5A, and RNA dependent RNA polymerase(rdrp) is coded by NS5B[4-5].

After the infection virus reaches to hepatocytes by an interaction between HCV E2 envelop glycoprotein and extracellular loop of CD81 on host. Where trans-membrane protein act as receptors expressed on surface of hepatocytes, B-lymphocytes, T-lymphocytes and natural-killer cells by CD81. Then it will binds to low density lipoprotein (LDL) receptors of

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dendritic cell-intracellular adhesion molecules-3-grabbing non-integrin (DC-SIGN)[4-5].

4. INHIBITORS FOR HCV

There has been a huge amount of drug development in hepatitis C with many new drug launched in the last decade. Hepatits C treatment used to consist of ribavirin and interferon which is generally pegylated, longer lasting form and which boost the body immune system [6].

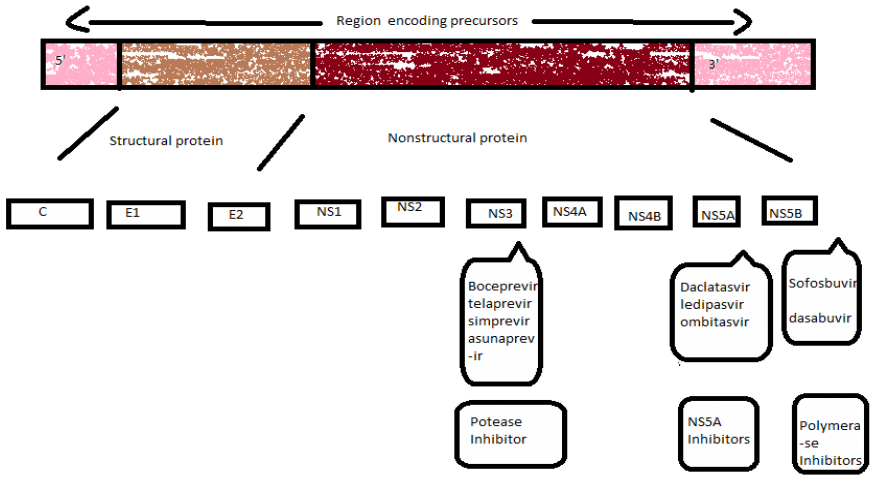

Fig 2: Protein encoded by Hepatitis C Virus genome as targets for direct acting antiviral agents

4.1. NS3 Protease inhibitors.

The HCV NS3 gene encodes for serine protease and NTPase/helicase. The NS4A gene encodes a co-factor for a serine protesae. Now the complex of NS3 and NS4A plays a major role in replication cycle specifically in maturation step. It blocks the activation of interferon regulatory factor3 (IRF-3) resulting in host immune evasion [16-17].

4.1.1. First wave HCV NS3 protease inhibitors Telaprevir and Boceprevir.

Telaprevir (TVR**)** is a peptidomimetic coumpound that inhibits the HCV complex NS3-4A serine protease whereas, Boceprevir(BVR) is a carboxamide – based compound and is also an oral HCV NS3 protease inhibitors. The bothe inhibitors bind to the active site of the NS3-4A serine protease enzyme reversibly. They prevent the cleavage of the portion of the genome derived polypeptide required for the generation of the individual non- structural proteins essential to the HCV viral life cycle. This is majorly adminstred by the addition of PEG-INF and RBV. Telaprevir and Boceprevir significantly improves virological outcome in treatment[11-12,15].

4.1.2. Second wave Protease Inhibitors.

Second wave protease inhibitors provides several advantages over currently available drugs. Semiprevir is a once a day oral NS3-4A protease inhibitors currently in phase clinical development for the treatment of HCV infection. Semiprevir is safe and well tolerated, by achieving SVR rate upto 92% when combine with sofosbuvir. It is effective in treating HCV genotype 1 which includes liver cirrhosis [13-14].

Asunaprevir (BNS-650032) is an inhibitor of HCV enzyme serine protease NS3. It is an experimental drug used for the treatment of hepatitis C. It is a twice- daily protease inhibitor being developed in both INF containing and free regimens with daclatasvir, an NS5A inhibitor and BMS791325, a non-nucleoside inhibitors. Asunaprevir is good protease inhibitor that decreases HCV replication activity and has an optimal rate of viral clearance [9-

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10].

Table 1 : HCV Protesase inhibitors[13]

Telaprevir	Approved	First generation
Boceprevir	Approved	First generation
Simeprevir	Phase 3	Second wave
BI1335	Phase 3	Second wave
Asunaprevir	Phase 3	Second wave
Danoprevir/r	Phase 2	Second wave
Sovaprevir	Phase 2	Second wave
MK 5172	Phase 2	Second generation
Ach 2684	Phase 2	Second generation

4.2. NS5B Polymerase Inhibitors.

The NS5B polymerase enzyme is an RNA dependent RNA polymerase that plays an essential role in HCV protein translation and synthesis leading to viral replication. It displays a finger/palm/thumb motif with the number of interaction site that are potential targets for drugs in development. There are currently two nucleoside analogs valopicitabine and viroPharma [9,16].

Valopicitabine(NM283) is a nucleoside analog that inhibits NS5B polymerase causing chain termination. Unfortunately, the clinical trials of this drug shows some risk profile due to which it has been temporarily placed on hold by the FDA [16].

ViroPharma(HCV-796) is a non nucleoside polymerase inhibitors that shows to be effective in early clinical trial. It has also been studied in combination with PEG-INF in a phase 1 [16].

Roche(R1626) is a nucleoside analog that inhibits the NS5B polymerase enzyme. It is a prodrug of R1479 (4'-acidocytidine) which selectively inhibits the HCV polymerase [16].

5. DIRECT ANTI-HCV AGENTS (DAA)

In 2011, the first generation of NS3/4A PIs (boceprevir and telaprevir) was approved for clinical use [12,15]. By the addition of boceprevir (BOC) or telaprevir (TVR) to PEG-IFN/RBV increases cure rate by 65%-75%. By 2013, the second generation of DAA drugs increases sustained virologic response (SVR) rates upto 90%-100% which also includes sofosbuvir [9,13-14].

5.1. First generation DAA's: boceprevir and telaprevir.

Boceprevir and telaprevir are HCV NS3/4A PIs and show potent inhibition of the HCV NS3/4A protease [9]. Meanwhile, combining with PEG-IFNα increases anti-HCV effects and decreases the emergency of resistance. These two drugs also show SVR rates increased 30%-40% with PEG-IFNα and RBV alone upto 65%-76% [11-12,17].

Table 2: Role of DAA in HCV guideline [9]

Drug regimen	**HCV genotype**
Sofosbuvir/ledipasvir+/-Ribavirin	Genotypes 1, 4, 5, and 6
Paritaprevir/ritonavir/ombitasvir+dasabuvir+/-ribavirin	Genotype 1
Sofosbuvir+ simeprevir+/-ribavirin	Genotypes 1 and 4

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Sofosbuvir+ daclatasvir+/-ribavirin	All Genotypes
Paritaprevir/ritonavir/ombitasvir+/-ribavirin	Genotype 4
Sofosbuvir+ ribavirin	Genotype 2 and 3

5.2. Second Generation DAAs

5.2.1. Sofosbuvir

Sofosbuvir is a nucleotide analog that inhibits HCV NS5B polymerase. It is converted to GS-331007 rapidly after injection. Later, GS331007 is taken up by hepatocytes and becomes GS-461203 active uridine analog 5'-triphosphate by cellular kinases. This triphosphate compound acts as the natural cellular uridine nucleotide which is incorporated by the HCV RNA polymerase into the elongation of RNA primer strand, results in chain termination. This drug increases SV R rate by 92%, combining with RBV SVR rate is of 100% for genotype 2. It is well tolerated and highly effective for patients those who are co-infected with HIV [14].

5.2.2. Simeprevir

Simeprevir is a highly specific and potent inhibitor of HCV NS3/4Aprotease. It is well distributed in liver. It is well tolerated and increases SVR rate by 79%-81%. When combined with sofosbuvir SVR rate is increased upto 92%. In 2013, FDA approved simeprevir for clinical use and for treating chronic HCV infection as a part of a triple antiviral treatment regimen [9,14].

5.2.3. Harvoni

Harvoni is a combination of fixed dose tablet containing ledipasvir and sofosbuvir for oral administration. Ledipasvir is an HCV NS5A inhibitor. The efficiency of Harvoni against HCV genotype 1 which increases SVR rates upto 93-99%. It was approved by FDA in 2014 [20].

6. MEDICINAL PLANTS AGAINST HEPATITIS C VIRUS

Medicinal plants have always caught the attention of researchers to develop drugs against fatal viral diseases. The current standard of care is a combination of pegylated interferon-α with ribavirin (RBV) and boceprevir /telaprevir. This treatment is partially effective but at the same time has significant side effects [19].

Table 3 : Medicinal plant phytochemicals as anti-HCV agents.

Phytochemicals	viral step	Effects
1.Diosgenin	Replication	Inhibition of transcription factor 3 and signal transducer
2.Silymarin/silibrin	Viral entry (viral attachment)	Inhibition of core protein and NS5 RDRP.
	Replication	RNA Dependent RNA Polymerase.
3.Iridoids	viral entry	Blockage of E2 and CD81 contact.
4.Narinenin	viral assembly	Suppression of core protein activity.
5.EGCG	viral entry	Disturbance in glycoprotein activity and inhibition of cell-cell transmission.
6.Ladanein	post attachment entry step	Inhibition of receptor interactions.

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6.1. Medicinal plants against HCV – Acetonic and methanaolic extracts of various plants have shown novel inhibition of HCV titer in-vitro confirmed by real time PCR. At present drug discovery is focused on medicinal herbs for HCV due to the lack of appropriate standard therapy. Plants likes *Acacia nilotic* [19].

6.2. Medicinal plant against HCV core – Plants like *Glycyrrhizin* which inhibits HCV titer dose and exhibits synergetic effects when administrated with interferon. Another plant *Silybun marianum* was tested for two pure fractions exhibited inhibition of HCV core protein of genotype 3A which was confirmed via western blotting. The combination of the plant with interferon shows promising result in treating HCV [19-20].

6.3. Medicinal plant agains NS3 Protease- Methanolic and water extract of medicinal plants such as *Boswellia carterii, Acacia nolitica, Syzygium aromaticum* have been tested against HCV protease and showed more than 90% at 100μg/ml. *Solanum nigrum* has also been tested against HCV and its methanolic and chloroform extracts exhibits inhibition against HCV protease in liver infected cells [20].

6.4. Medicinal plant phytochemicals as HCV Inhibitor – Currently there are many plants derivatives been tested against HCV which shows significant inhibition in entry, replication, an assembly steps of viral life cycle [20-21].

Diosgenin (3β-hydroxy-5-spirostene) is a plant derived sapogenin which effectively blocks the replication of HCV subgenomic replicon system at both mRNA and the protein level [19].

Silymarin is isolated from Silybun marianum has been tested against HCV and is found to be effective in inhibiting viral activity of HCV [19-22]

7. CONCLUSION

Hepatitis C is a liver disease that can have serious consequences at times fatal. Treatment of HCV infection is undergoing a revolution. Interferon based combinations had been there for about 24 years but now is being replaced by Interferon free therapies and many direct anti-viral agents(DAA). There are various drugs which forms complex with PEG-INF+RBV Which helps in anti-HCV therapies. Major drugs are Boceprevir, telaprevir, asunaprevir, semiprevir, etc. The combination of sofosbuvir and velpatasvir resulted in cure rate about 99%. The development of protease inhibitors and limitation of first generation PIs have significantly lead to various drug development and efficacy of HCV treatment.

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REPURPOSING ESSENTIAL OIL FOR CONTROL OF PHYTOPATHOGENIC FUNGI

Ramesh Kumar Kushwaha[1,*], Varsha R[1], Nikhil Kalshetty[2], C.S. Vivek Babu[2]
[1]Department of Biochemistry, School of Applied Science, REVA University, Bangalore, 560064, India
[2]CSIR-Central Institute of Medicinal and Aromatic Plants, Research Centre, Allalasandra, GKVK Post, Bangalore - 560 065, India.

ABSTARCT: Several synthetic fungicides are available in market to control the phytopathogen that resulting into emergence of varietal fungicide resistant fungal pathogen. Apart from, persistent natures of synthetic fungicide are leading to deleterious effect on animal and human beings. In this context, we continued the application of lemon grass and Eucalyptus oil to destructive pathogens *Sclerocium rolfsii*, *Colletotrichum gloeosporioides* and *Fusarium oxysporum*, having wide range of host they cause significant product loss in variety of agricultural products ranging from vegetables, fruits to cereals and some pulses. Agar well diffusion method was used for assay, 100% pure lemon grass and Eucalyptus oil, applied agar well in range of 20µl, 15µl, 10µl, 5µl, 5µl, 2.5µl, 1.25µl and 0.625µl to inhibit the pathogen. ED50 for lemon-citronella are 1.25µl and 5µl for *S. rolfsii* and *F. oxysporum* respectively, however ED50 for Eucalyptus oil was 5µl for both *S. rolfsii* and *F. oxysporum*. Different concentration (0.01% to 1.0% v/v) of lemon grass and Eucalyptus oil were applied to *Solanum lycopersicum* and *Ocimum* species to check the effect on seed germination. 0.1% and 0.05% (v/v) lemon oil was sufficient for the seed germination of *Osmium sanctum* and *O. basillicum* respectively. 0.1% (v/v) Eucalyptus oil was sufficient for the inhibition *S. lycopersicum* seed germination. Therefore, the essential oil can be used as ecofriendly formulation against the fungal pathogens or can be used to germination inhibitory agent for seed as economic value.

Keyword: *Sclerocium rolfsii*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Solanum lycopersicum*, *Osmium sanctum*, *O. basillicum*

***Correspondence author:** Ramesh Kumar Kushwaha
Department of Biochemistry, School of Applied Science, REVA University, Bangalore, 560064, India. Email:- kush0109@yahoo.com

1. INTRODUCTION

Medicinal and aromatic plants (MAPs) are very important for human being to used as pharmaceutical, flavor, fragrance, perfumery, and cosmetic industries, which has been using since from ancient time. Due to side effect of synthetic compound, at present scenario global population are moving toward traditional plant-based medications for treating various human health problems. Every year increasing the demand of herbal productand expected demand grow up to 5 trillion USD by the year 2050 [1]. MAPs are important source of natural essential oils which have unique flavor and fragrance properties and also biological activities. Essential oil contains the numerous chemical groups, such as terpenes, aldehydes, alcohols, esters, phenolic, ethers, and ketones [2]. Eucalyptus, Camphor, and Lemongrass essential oil reported to inhibit the seed gemination *of Parthenium hysterophorus* [3].

Cymbopogon flexuosus or lemon grass oil has been traditionally used as a remedy for a variety of health conditions and scientifically proved its significances as analgesic, antiinflammatory, antipyretic, diuretic and sedative well known insect repellent contains [4]. Lemongrass (*C .flexuosus*) oil is characterized for monoterpenes compounds, which

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constitute the major compound citral-a (33.1 %), citral-b (30.0 %), geranyl acetate (12.0 %) and linalool (2.6 %) [5]. Genus *Cymbopogon* belong to numerous species and dominant compound is citral (3,7-dimethyloct-6-en-1-al) and has been used as a topical insect repellent in children and other sensitive populations under the US EPA (Environmental Protection Agency) [6]. Lemongrass oil has been also reported as the antimicrobial and allelopathogenic activity against pathogen and inhibit the growth of others plant seed [7].

Eucalyptus citriodora, or Corymbia citriodora oil are traditionally used as analgesic, anti-inflammatory, antipyretic and remedies for respiratory infections (cold, flu, and sinus). Beside that, eucalyptus oil is well known antimicrobial and insecticidal activity which inhibits the microbial pathogens and kills insect respectively [8-9]. The chemical constituent in eucalyptus oil is dominated by the citronellal (69.77%), citronellol (10.63%) and isopulegol (4.66%). However, decaying leaf oil and its prominent two major components (citronellal and citronellol) are known to inhibit the seed germination and root growth inhibition i.e showed allelopatic effects on some others plant [10].

Sclerotium rolfsii is a necrotrophic, soil borne fungal pathogen, causes disease on hundreds of plant species, including field, vegetable, fruit, and ornamental crops. At global scenario *S. rolfsii* causes to loss of 10-20 million dollars because loss of 1-60% yield at field condition [11]. *Colletotrichum gloeosporioides* (Anthracnose) is one of the most common *colletotrichum* fungal plant pathogen and causes bitter rot in variety of crops worldwide [12]. This fungi cause the anthracnose disease in fruit and vegetable, which the major loss [13]. Another soil born pathogen belongs to ascomycete called *Fusarium oxysporum* that cause fusarium wilt (a deadly vascular wilting syndrome in plants) in most of plant worldwide [14].

In context of the present scenario to control the fungal pathogen by the natural compound, studied the application of lemon grass and eucalyptus oil against the fungal pathogen *S. rolfsii*, *C. gloeosporioides*, and *F. oxysporum*. Also the same concentration of essential oil was applied to tomato and ocimum seed to validate the effect of essential oil on nursery or plant development.

2. MATERIALS AND METHODS

2.1 Essential oil material collection

Pure freshly distilled eucalyptus and lemon grass oil were collected from distillation unit head of CSIR-Central Institute of Medicinal and Aromatic plants, Research Centre, Bangalore (India).

2.2 Pathogenic fungal strains

All pathogens were isolated from the infected medicinal plant from research field of CSIR-CIMAP, Research Centre, Bangalore. Pure cultures of all pathogens were identified and maintained in the lab for experiments. Here, three potent pathogen *S. rolfsii*, *C. gloeosporioides*, and *F. oxysporum* were considered.

2.3 Antifungal assay

Antifungal activity assay of eucalyptus and lemon grass oil were followed by the agar well diffusion method [15]. Agar well was prepared in PDA plate though 6mm diameter corkborer and pathogen agar plug (6mm diameter plug from actively growing mother culture plate) kept on center of PDA plate. Apart from 100% concentration, different concentration of eucalyptus or lemon grass oil were prepared through diluent (0.5% T20 in distilled water) such as 75%, 50%, 25%. 20µl from each concentration of eucalyptus or lemon grass oil like 100%, 75%,

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50% and 25% was inoculated in agar well, fungicide mancozeb and copper sulphate were considered as positive control.

2.4 Effect of essential oil on seed germination

O. basilcum, *O. sanctum* and *Solanum lycopersicum* seed soaked in water for one hour and arrange on wetted blotting sheet at 1.0 cm down from top, thereafter folded in round manner. Seed containing folded blotting paper dipped ¾ part in test tube containing different solvent such (1) pure autoclaved water (2) autoclaved water containing 0.5% T20 (3) different concentration of essential oil as represented in figure legends. All tubes were kept at root temperature under natural condition and were observing the germination every day.

2.5 Statistical analysis

Three numbers of biological replicates were considered in each experiment. Calculations of mean, standard deviations or standard errors for each data were used for statistical evaluation using social science statistic online software. The statistical significance of differences between control and treated samples were tested by unpaired Student's t-test.

3. RESULTS AND DISCUSSION

3.1 Effect of essential oil on the pathogen growth

Lemon grass oil was successful in inhibiting two fungal pathogens *S. rolfsii* and *F. oxysporum* but the *C. gloeosporiodies* seemed to be a resistant strain (Fig.1b). The mechanism of action well not studied it could be mentioned that few volatile compounds present in the oil could be one of the key reasons that essential oils have antimicrobial activity. Upon screening which gave almost 100% results for standard 20µl of sample and further tested for its effective dose (ED). Effective dose test for the essential oil of lemon grass oil against the three-fungal pathogen was validated, for *S. rolfsii* seemed to be 1.25µl and for *F. oxysporum* it seemed to be 5µl while *C. gloeosporiodies* remained resistant strain. Previously reported the application of *Cymbopogon citratus* oil was used to inhibit the growth of *Aspergillus* species pathogenic fungi [16]. Apart from the lemongrass oil, so many others essential are reported for the antifungal activity [17-18].

Next essential oil was eucalyptus oil, which is lesser dense oil than lemon grass oil and having more volatile with strong aroma. Initially, 20µl pure oil gave positive results; it was further tested for its ED values. As depicted in figure 1c, the essential oil was successful in inhibiting two fungal pathogens *S. rolfsii* and *F. oxysporum* but the *C. gloeosporiodies* seemed to be a resistant strain. Effective dose test for the essential oil of eucalyptus against the two fungal pathogens *S. rolfsii* and *F. oxysporum* were 5µl while *C. gloeosporiodies* was remained resistant. Although eucalyptus oil is a much lighter than the lemon grass and much more volatile it seemed to be effective at concentrations higher than that of lemon grass. Mehani and coworker have been proved the eucalyptus oil antifungal activity against the *F. graminearum*, *F. sporotrichioide* and others *Fusarium* species [19-20].

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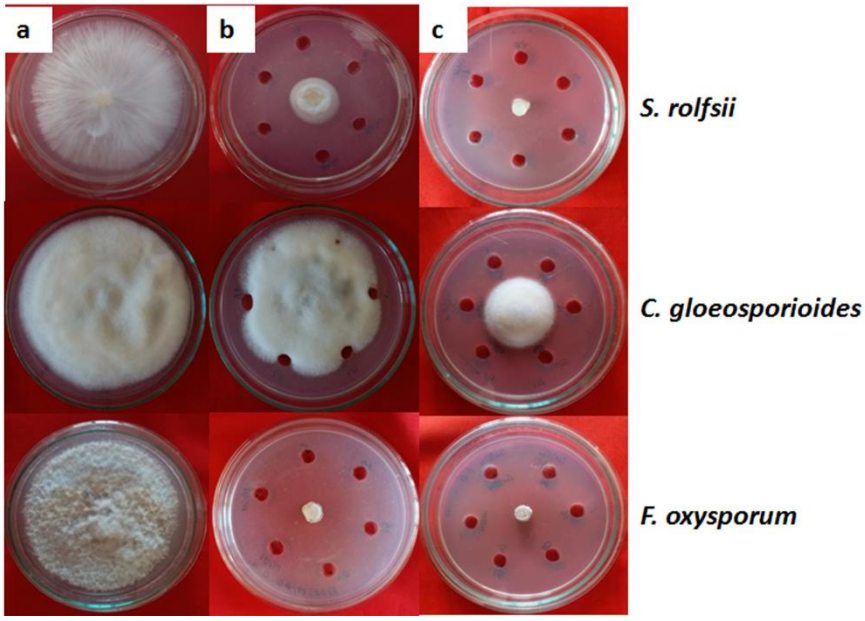

Figure 1. Effect of essential oil on *S. rolfsii*, *C. gloeosporioides*, and *F. oxysporum* growth (a) Control (b) different concentration of lemon grass oil (c) different concentration of eucalyptus oil

3.2 Effect of essential oil on seed germination and nursery development

Further application of essential oil was validated for their effect on seed germination and seedling development. The procedure for essential oil treatments were followed in soil as well as on blotting paper in test tube as shown in figure 2. The concentration of both oil that is above 0.1% or 5µl in 10ml of the solution would have negative impact on breaking seed dormancy and in shooting and rooting (Fig. 3 & 4). It is also evident that the same concentrations in soil have lesser effect on seed germination than in the tubes (Fig. 2). It was also observed that plants which grew after the treatment with the EO had a thicker and shorter shoot system and these plants were get least viral infection compared with untreated plants. So, might be EO may act as immune inducer to the plant when applied on early stage of its life to give it a stronger immune response.

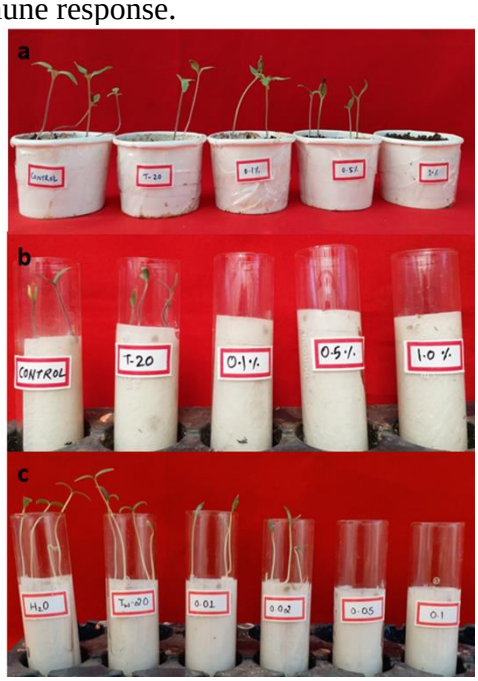

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Figure 2. The experiment performed as (a) *S. lycopersicum* grown in soil under treatment of lemon grass oil (b) *Ocimum sanctum* grown in tube under treatment of lemon grass oil (c) *Ocimum basalicum* grown in tube under treatment of lemon grass oil

Since the EOs are natural products of plant system can be used to immunize the soil after harvesting the crop the land scape through mulching with the waste or left-over parts of these EOs source plants. In this regard, the whole plant canbe utilized and aids to help in protecting agricultural crops.

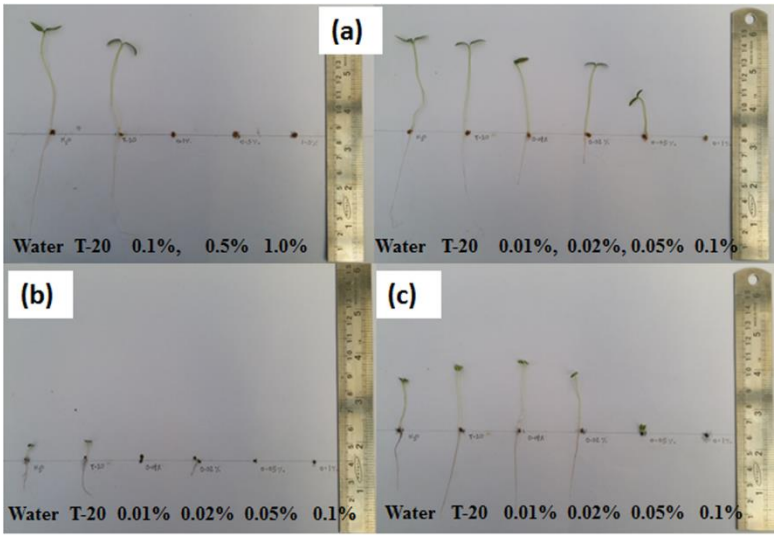

Figure 3. Effect of lemon grass oil on seed germination and seedling development of (a) *Solanum lycopersicum* (b) *Ocimum sanctum* (c) *Ocimum basalicum*

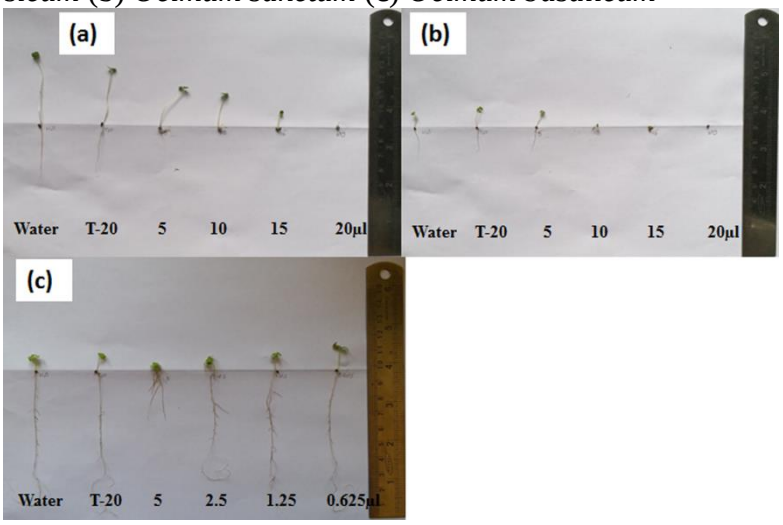

Figure 4. Effect of eucalyptus oil on seed germination and seedling development of (a) different concentration of oil in 10ml water (0.5% T-20) *Ocimum basalicum* (b) & (c) different concentration of oil in 10ml water (0.5% T-20) *Ocimum sanctum*

As we found effect of the essential oil of lemon grass on seed germination in *Solanum lycopersicu*m (tomato) more than 0.1% to hindering seed germination this poses as a threat if one wants to use the oil in coating of seed for pathogen treatment. Since the oil can not be used in these concentrations and reduced from 0.1%. 0.5% and 1.0% to 0.01%, 0.02%, 0.05% and 0.1%. to check seed germination rate and results were better than preceding concentrations. Thereafter was tested on two different *Ocimum* species, *O. sanctum* and *O. basillicum* with concentrations ranging from 0.01% to 0.1%. Unlike previous results the germination rate was slower and the number of seedlings that emerged successfully were less

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in number. Among these two different *Ocimum* species, *O. Sanctum* is much more resistant to the negative effects of the essential oil against its germination rate that *O. basilicum* which showed very sensitive results having almost no germinations at all except for at the concentration of 0.02% (Fig.3).

At high concentration of essential oil was acted as allelopatic i.e not suitable for the priming of seed with high concentration essential oil. However at low concentration of essential oil was good for the immunizing the seedling with little compromise with growth. Earlier studied the essential oils eucalyptus, camphor, and lemongrass were used different concentration (0 to 20 ml/l) to inhibit the *Parthenium hysterophorus* seed germination [3]. As compared with *O. basilcum* and *S. lycopersiucm*, *O. sanctum* is more sensitive at same concentration of lemon grass oil (Fig.3a-c). Similarly, eucalyptus oil also inhibited the shoot and root length on *Ocimum* species, while at lower concentration of eucalyptus oil, induced more growth of root length in *O. sanctum* plant (Fig.4a-c).

The results were lemon grass at the oil concentration of 5µl, 2.5µl, 1.25µl and 0.625µl could germinate at a normal rate in comparison to the controls and the number of seedlings emerged were almost about 80% (Fig. 5e,f). Lemon grass oil was significantly decreased the shoot and length all three tested plant (Fig. 5a-f), however eucalyptus oil showed negative effect on the shoot and root after 10µl (Fig. 6a-b). Shokouhian et al., 2016 have applied three essential oils rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*) and anise (*Pimpinella anisum*) to seeds of lettuce (*Lactuca sativa*), pepper (*Piper longum*) and tomato (*Solanum lycopersicum*) at different concentration which inhibited the seed germination [21]. Since the eucalyptus oil has a higher ED value so the treatment dose was raised from 5µl to 20µl in 10ml of carrier, 20µl being the highest concentration gives zero results so does 15 and 10µl concentrations but 5µl being the lowest concentration has little effect on the germination in comparison to the controls used which were water and the carrier used to prepare the oil solution.

Because of allelopathy activity of essential oil, can be used as a model for natural herbicide actions to control the weed and pest management [22-23]. Apart from essential oil, natural phytohormone like absicic acid have capacity to inhibit the germination or prolong the seed the germination [24].

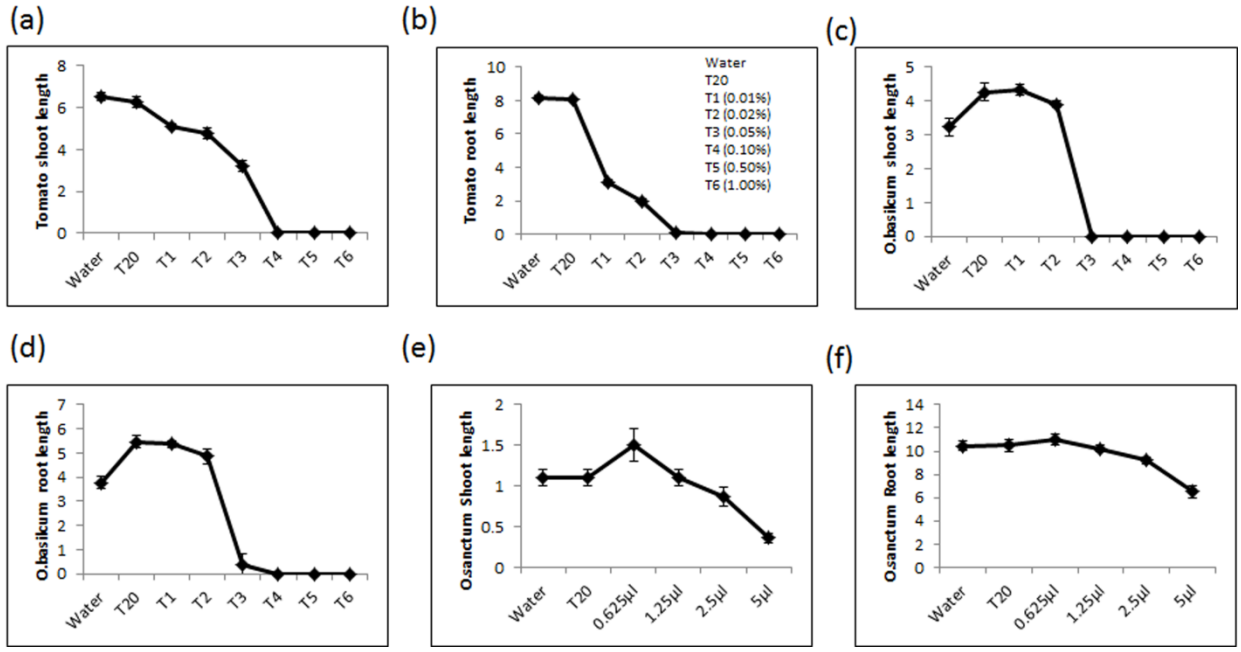

Figure 5. Effect of lemon grass oil (a) tomato shoot length (b) tomato root length (c) *O.*

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basilcum shoot length (d) *O. basilcum* root length (e) *O. sanctum* shoot length (conc. Of oil in 10 ml of T-20) (f) *O. sanctum* root length (conc. Of oil in 10 ml of T-20).

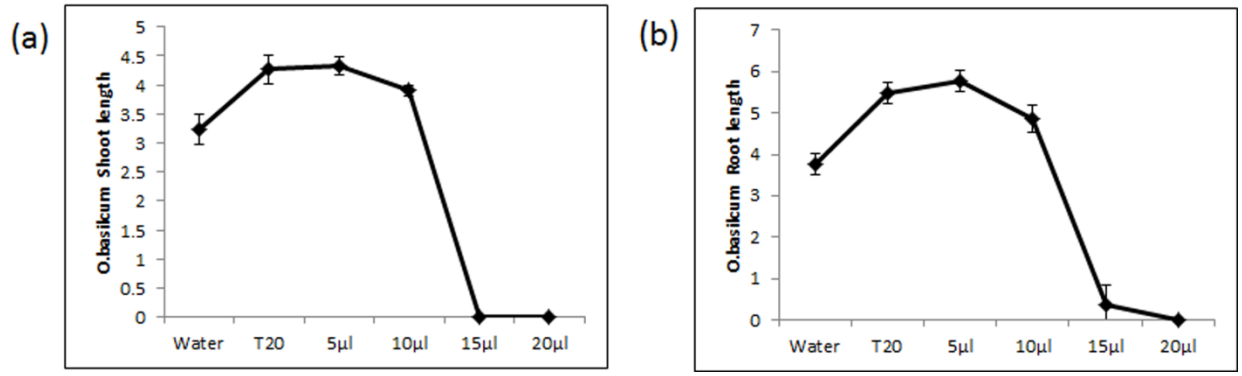

Figure 6. Effect of different concentration of eucalyptus oil in 10ml carrier material (0.5% T20) (a) *O. basilcum* shoot length (b) *O. basilcum* root length

4. CONCLUSION

In this study, we performed the antifungal activity of essential oil (eucalyptus and lemongrass oil). Here, we observed that both essential oil showed an interesting biological activity on *S. rolfsii* and *F. oxysporum*. Antifungal studies confirmed the effectiveness of essential oils against microorganisms studied. In addition antifungal also showed anti seed germination activity on tomato and *Ocimum* species. The application can be further extended to the field of medicine where probably this can be used as application formula in controlling the common fungal infections. And in agriculture field probably a aerosol version of this EO could be a greater use in controlling the dangerous fungal pathogens like *S. rolfsii* and *F. oxysporum*.

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COMPUTATIONAL SCREENING OF ENANTIOMERIC LIGNANS AND NEOLIGNANS FROM *PHYLLANTHUS GLAUCUS* FOR INHIBITION OF DENGUE VIRAL PROTEASE AND HEPATITIS C VIRUS

Pooja R Karkera[1] and Ravikumar Y S[2]*
[1]Department of Biochemistry, REVA University, Bangalore-560 064, Karnataka, India
[2]Department of Biotechnology, M.S. Ramaiah Institute of Technology, Bangalore-560 054, Karnataka, India

ABSTRACT: Hepatitis C virus (HCV) and Dengue virus (DENV) are one of the leading viral diseases which are globally prevalent causing complex health conditions. While HCV targets the hepatocytes and caused severe liver complications, DENV causes Dengue fever and dengue hemorrhagic fever/dengue shock syndrome. Non-structural protein NS3 protease plays a major role in the processing of polyprotein and replication of both these viruses. NS5b is the RNA dependent RNA polymerase which is also a key enzyme in the replication of the virus. Hence, these proteins can be targeted to inhibit the virus. Unfortunately, there is no fool proof treatment to inhibit these viruses till date. In this view, we selected 12 Enantiomeric Lignans and Neolignans from *Phyllanthus glaucus;* docked it against two serotypes (3L6P & 2VBC) of DENV NS3 protease and the active sites of 2 proteins (NS3 & NS5B) of HCV to obtain the lead molecules. (+) -(7R,8S)-phyllanglaucin A showed best interaction against 2VBC genotype of DENV and the NS5B protein of HCV. Whereas, (+)-phyllanglaucin B showed best interaction with NS3 protease of HCV and (−)-phyllanglaucin C had the least binding affinity with the 3L6P genotype of DENV. However, *In vitro* studies have to be performed to confirm the pharmacophore properties of these lignans.

Key words: DENV, HCV, protease, molecular docking

***Correspondence author:** Ravikumar Y S. Email: ysravish@gmail.com
Department of Biotechnology, M.S. Ramaiah Institute of Technology, Bangalore-560 054, Karnataka, India

1. INTRODUCTION

Hepatitis C virus (HCV) and Dengue virus (DENV) are one of the leading viral diseases which are globally prevalent causing complex health conditions. HCV affects the liver cells i.e. the hepatocytes which are majorly involved in removal of toxic substances from blood [1] and causes complex liver diseases including advanced fibrosis, cirrhosis and hepatocellular carcinoma [2]. DENV has four genotypes; all of which causes three kinds of diseases in the increasing order of severity starting from Dengue fever, Dengue haemorrhagic fever and dengue shock syndrome [3]. Both the viruses cause infections which do not have a fool proof treatment yet as the treatment should be capable of curing the patient affected with any of the virus's genotype. There is no suitable licensed vaccine against DENV [4]. Enormous efforts have been made since several years to come up with inhibitors against all the genotypes of HCV and DENV [5]. Due to lack of animal models and low reactogenicity, it's been a tough situation to combat these viral diseases with an effective treatment [4].

Both DENV and HCV have single stranded, positive sense RNA which is around 11kb and

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9.6kb respectively; both belong to the Flaviviridae family [6, 7]. It further translates into a polyprotein which is internally cleaved and processed into ten mature proteins which include both structural and non-structural proteins [8, 9]. Core protein C, envelope glycoproteins (E) and transmembranal ion protein are the structural proteins synthesised by both the viruses [10]. While, HCV genome translates to NS2, NS3, NS4A, NS4B, NS5A and NS5B non-structural proteins [11]; DENV translates to NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [12]. Non-structural proteins play a major role in replication of the virus in the host organism. HCV NS3 has a serine protease and a helicase situated on either ends [13]. Similarly, DENV NS3 also exhibit protease and helicase activity. NS3 forms the major target in both the viruses as the protease is responsible for making the other proteins functional. NS5 in DENV and NS5B in HCV being the RNA dependent RNA polymerase forms another major target for inhibition of viral replication [14, 15].

Viral genome unlike bacteria is subjected to rapid mutations and a new drug has to be developed at regular intervals [16]. This makes the treatment process very expensive which is impossible to be afforded by developing countries. Plant sources have been used to treat several diseases since ancient times. Plants are the indirect or direct source of half of the approved drugs [17]. Plants belonging to the genus Phyllanthus have been used to treat kidney and urinary bladder disturbances, intestinal infections, diabetes and hepatitis B since a very long time [18]. Enantiomeric Lignans and Neolignans were isolated from whole plant extracts of *Phyllanthus glaucus* [19]. In view of this, we have investigated the anti-viral activity of these lignans by targeting against two serotypes of NS3 protein of DENV and against the active sites of two proteins i.e. N3 and NS5B of HCV.

2. MATERIALS AND METHODS

2.1 Construction of library of Compounds

Enantiomeric lignans and neolignans which have been isolated from 70% acetone extract of the whole plants of *Phyllanthus glaucus* wall by [19] were used for the study.

2.2 Molecular Docking

Molecular docking of these compounds against NS3 and NS5B protein of HCV and 2 serotypes of DENV was carried out following these steps

2.2.1 Preparation of Ligands

The 2D structure of the molecule was drawn in a ChemDraw software and saved as a mol file. 2D structure was converted into 3D structure by optimizing the energy of the molecule on Avogadro software and saved as a pdb file. This was further optimized by setting torsion and making the number of rotatable bonds as nil in them on AutoDock software and saved as a pdbqt file.

2.2.2 Preparation of Proteins

The structure of NS3 protein from two serotypes of DENV was downloaded from the Protein Data Bank (PDB) using the PDB ID 2VBC and 3L6P. Structure of NS3 protein of HCV was downloaded using PDB ID 1DY8A and NS5B structure of HCV was downloaded using the PDB ID 3TYVA. These structures were opened one at a time in Autodock software; the water molecules and heteroatoms were removed to optimize the molecule. Grid box having confirmation: (center_x = 69.6242422859 center_y = -19.4726972842 center_z = 0.732205659307) was chosen for NS3 protein (1DY8A) on its active site His57, Ser139, Arg155, Ala157, Ala156 and center_x = 24.5043099088 center_y = 46.3929281386 center_z = 40.4721285987 was chosen for NS5B protein of HCV (3TYVA) on its active site Pro 197, Tyr448, Leu384, Met414, Arg 200 and Tyr 415. Grid box conformation for NS3 protein of two serotypes of DENV were center_x = -8.3198314043 center_y = 1.8548080499 center_z =

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19.4967101113 for 2VBC on its active site Leu-30, His-51, Glu-61, Thr-62, Trp-69, Asp-75, Lys-104, Ser-135, Asn-152, Ser-158, Gln-167, Glu-173 and center_x = -21.4066 center_y = -17.3464 center_z = 9.94350433252 for 3L6P on its active site Ile-75 ,Arg-104, Gly-105,Pro-117,Trp-119, Asp-125, Tyr-129,Gly-130,Gly-131, Trp-133, Phe-135, Trp-139, Pro-152, Gln-160, Pro-163, Gly-174, Asp-179, Gly-183, Ser-185, Tyr-200, Gly-201, Asn-202, Gly-203, Val-212, Ser-213, Ile-215, Gln-217.

2.2.3 Molecular Docking

The pdb files of the protein molecule and the ligand was docked together in the AutoDock software; with one ligand against one protein at a time using the command prompt. The results will show 10 models of confirmation for the protein and ligand interaction. The model having the least binding affinity will be analysed and visualized further using Ligplot software [10]. The same procedure is repeated for all the four protein molecules.

3.RESULTS

3.1 List of Compounds

A library of 12 compounds was constructed for this study.

Table1: List of compounds

Sl No.	No.	Compound Name
1	10	(7S,8R)-cedrusin
2	12	(7S,8R)- 4,7,9,9′ -tetrahydroxy-3,3′ -dimethoxy-8-O-4′ -neolignan
3	1a	(+) -(7R,8S)-phyllanglaucin A
4	1b	(−) -(7S,8R)-phyllanglaucin A
5	2a	(7R,8R)-4,4′ -dihydroxy-3,7,3′ -trimethoxy-8,1′ -7′ ,8′ ,9′ -trinor-neolignan-9-ol
6	2b	(7S,8S)-4,4′ -dihydroxy-3,7,3′ -trimethoxy-8,1′ -7′ ,8′ ,9′ -trinor-neolignan-9-ol
7	4a	(+)-phyllanglaucin B
8	4b	(−)-phyllanglaucin B
9	5a	(+)-phyllanglaucin C
10	5b	(−)-phyllanglaucin C
11	7a	(7R,8S,7′ R,8′ R)-acernikol
12	7b	(7S,8R,7′ S,8′ S)-acernikol

3.2 Results obtained from docking study

Leads molecules against NS3 protease (1DY8A) HCV

4A showed the best interaction with NS3 protease with a binding affinity of -17.1 kcal/mol. The compound showed hydrophobic interactions with 7 amino acid base of the active site including Val 107, Val133, Val35, Ser7, Gln8, Asp30 and Gly31 and hydrophilic interaction with Thr108. 5B showed the second best interaction with the protein having a binding affinity of -16.8 kcal/mol. Here the compound has two hydrophilic interactions with Ser20 and Gln8 and 8 hydrophobic interactions including Thr19, Ala65, Val35, Arg11, Thr10, Gln34, Gln 9, Cys16. Figure 1 represent the molecular model of docked conformation of lead compounds on NS3 protein active site of HCV.

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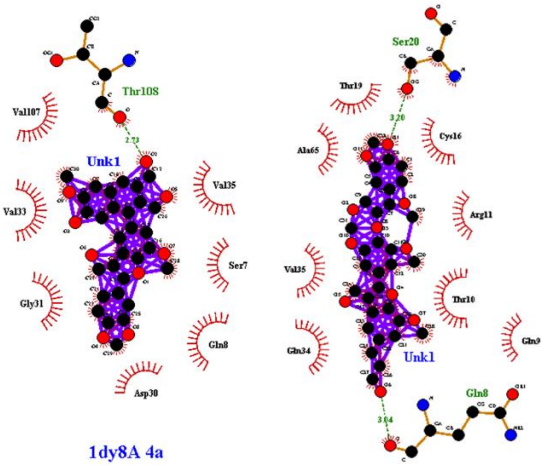

Fig 1: Interaction of 4a and 5b with HCV NS3 protease

Leads molecules against NS5B (3TYVA) of HCV

1a showed best interaction with NS5B protein of HCV with a binding affinity of -16.4 kcal/mol and showed interactions with 10 amino acids including Val381, Lys198, Pro417, Pro197, Ser470, Val201, Leu384, Tyr383 (hydrophobic) His467, Tyr382 (hydrophilic). This is followed by 5B which has shown very good interaction with NS5B with a binding affinity of -16.2 kcal/mol. The compound exhibited hydrophobic interactions with Ser556, Phe193, Tyr448, Asn316, Cys366, Leu384, Pro197, Tyr415 and a single hydrophilic interaction with Met414. Figure 2 represent the molecular model of docked conformation of lead compounds on NS5B protein active site of HCV.

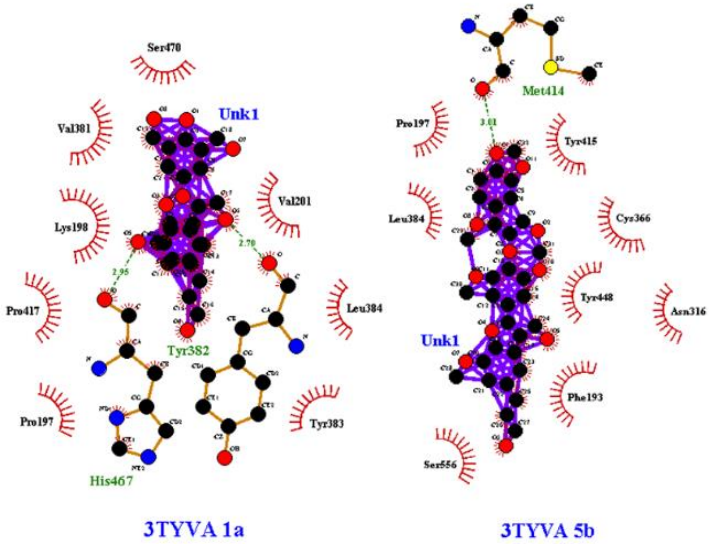

Fig 2: Interaction of 1a and 5b with HCV NS5B

Leads molecules against NS3 (3L6P) protease of serotype type 3 DENV

Here again 5b showed the best interaction with a binding affinity of -14.9 kcal/mol. It showed hydrophobic interactions with Leu176, Val212, Tyr200, Gly203, Tyr211, Ser213, Ser185, Asn202, Val86, Gly183, Pro182 and hydrophilic interaction with Gly201. 1a showed the second best interaction with a binding affinity of -14.8 kcal/mol. It showed hydrophobic interactions with Ile30, Trp17, Asn191, Arg192 and more hydrophilic interactions with Asn29, Leu31, Gln160, Glu144, Glu143, Glu193. Figure 3 represent the molecular model of

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docked conformation of lead compounds on NS3 protein active site of serotype type 3 DENV.

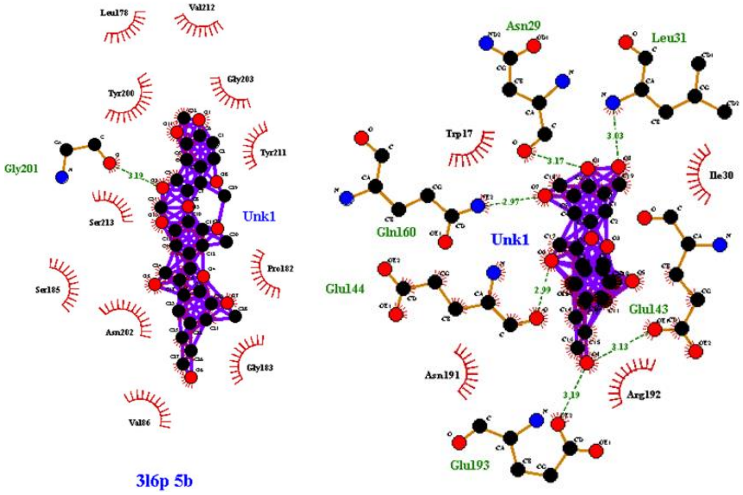

Fig 3: Interaction of 5B and 1A with NS3 (3L6P) protease of serotype type 3 DENV

Leads molecules with NS3 (2VBC) protease DENV serotype type 2

1a showed best interaction with NS5B protein of HCV with a binding affinity of -17.2 kcal/mol. It shows hydrophilic interaction Ser453, Asp409, Thr289, Thr450, Lys430, Asn481, His485, Asp482 and hydrophobic interaction with Cys428, Val449, Pro448. 5b showed the second best interaction -16.7 kcal/mol interacting hydrophobically with Glu468, Gln471, Gly420, Gln467, Asp469, Ala466, Asn464 and hydrophilically with Tyr354, Asp470, Ala419, Arg418. Figure 4 represent the molecular model of docked conformation of lead compounds on NS3 protein active site of serotype type 2 DENV.

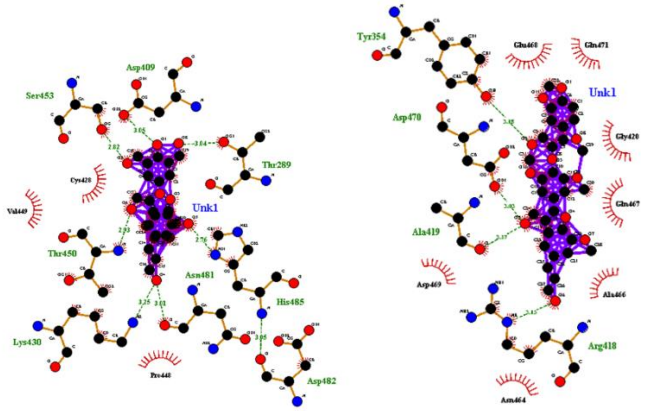

Fig 3: Interaction of IA and 5B with NS3 (2VBC) protease of serotype type 2 DENV

Table2: Binding energy of compounds against DENV and HCV proteins

Sl. No.	Lignins	Binding energy [-kcal/mol] against the HCV and DENV proteins			
		Dengue Virus NS3 protein		Hepatitis C Virus	
		3L6P	2VBC	NS5B 3TYVA	NS3 IDY8A
1	10	11.6	12.7	11.9	11.9
2	12	11.7	13.1	12.1	12.7
3	1a	14.8	17.2	16.4	15.7
4	1b	11.9	14.0	12.0	12.7

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5	2a	9.9	12.5	11.4	11.3
6	2b	10.3	11.8	10.5	11.1
7	4a	14.5	16.0	15.7	17.1
8	4b	14.3	16.4	16.0	16.8
9	5a	14.5	16.3	15.7	16.7
10	5b	14.9	16.7	16.2	16.8
11	7a	14.8	17.1	15.4	16.6
12	7b	14.7	16.4	16.1	16.5

4. DISCUSSION

HCV and DENV being leading viruses in causing complex diseases which don't have a proper prophylaxis till date. Many efforts have been made in this aspect to come up with a drug that inhibits all the genotypes of the viruses. N number of plants have been tested for their medicinal properties especially anti-viral properties and many plants have already been used as anti-viral agents. The mechanism with which all the plants work is similar and the metabolism of plant products in the body is easier when compared to synthesized compounds [20]. The cost involved in developing treatment using plants is very economical. Thus, everyone is now targeting on developing plant based treatment like those present in *Phyllanthus* which is known for a lot of remedies [21].

In this study, we selected 12 enantiomeric lignins that were isolated by [19] from *Phyllanthus glaucus* based on the significance the genus is already reported for (Table1). This was docked against serotype 2 (2VBC) and 3 (3L6P) of NS3 protease of DENV. It was also docked against NS3(IDY8A) protease and NS5B (3TYVA) of HCV. In this process, the Auto dock Vina software is in search of that confirmation between the ligand and the protein which exhibits the least binding energy in them and thus is considered to be most stable among the rest. The software gives a total of 9 models in the order of the best ranks. Making it obvious, the model that is ranked first will have the least binding affinity among them and will be selected for the further steps. For example, docking of (−)-phyllanglaucin C with NS3 protease of HCV [IDY8A]: 1-9 models with the binding affinity of 16.8, 16.6, 16.4, 16.1, 16., 16.1, 15.9, 15.8, 15.7 (-kcal/mol) respectively. The model having the affinity of -16.8 kcal/mol will be selected for further merging with the protein and visualizing it on the Ligplot software. (−)-phyllanglaucin C (5b) has shown good interaction against the two proteins of HCV and two proteins of DENV followed by (+) -(7R,8S)-phyllanglaucin A (1a). 1a has shown best interaction against 2VBC genotype of DENV and the NS5B protein of HCV. Whereas, (+)-phyllanglaucin B showed best interaction with NS3 protease of HCV and (−)-phyllanglaucin C had the least binding affinity with the 3L6P genotype of DENV. 5b has consistently shown good binding affinity with both serotypes of NS3 of DENV. It also shows good interaction with NS3 and NS5B of HCV. It had a binding affinity of 14.9, 16.7, 16.2, 16.8 (-kcal/mol) with 3L6P and 2VBC of DENV, 3TYVA (NS5B) and IDY8A (NS3) of HCV respectively.

Visualization of the docked confirmation can be observed by merging the best ranked model with the pdb file of the protein and opened in the Ligplot software. This lets us visualize the interaction of the lignins with the amino acid residues in and around the active site of the protein. In the output, the lignin will appear in purple colour, the amino acid residues appear in red when there is a hydrophobic interaction with the ligand; the hydrophobic interactions are represented as a semi circled structure, for the hydrophilic interactions the amino acid residues are represented in green. The bond lengths between the oxygen atoms are also represented. 1a having best interaction with serotype 2 of DENV showed hydrophilic interaction Ser453, Asp409, Thr289, Thr450, Lys430, Asn481, His485, Asp482 and

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hydrophobic interaction with Cys428, Val449, Pro448. 5b showed the second best interaction -16.7 kcal/mol interacting hydrophobically with Glu468, Gln471, Gly420, Gln467, Asp469, Ala466, Asn464 and hydrophilically with Tyr354, Asp470, Ala419, Arg418. It also shows best interaction with NS5B of HCV interacting with 10 amino acids including Val381, Lys198, Pro417, Pro197, Ser470, Val201, Leu384, Tyr383 (hydrophobic) His467, Tyr382 (hydrophilic). 4A showed the best interaction with NS3 protease with a binding affinity of -17.1 kcal/mol. The compound showed hydrophobic interactions with 7 amino acid base of the active site including Val 107, Val133, Val35, Ser7, Gln8, Asp30 and Gly31 and hydrophilic interaction with Thr108. 5b showing the second best interaction with serotype 2 of DENV, NS3 and NS5B of HCV interact hydrophobically with Glu468, Gln471, Gly420, Gln467, Asp469, Ala466, Asn464 and hydrophilically with Tyr354, Asp470, Ala419, Arg418; has two hydrophilic interactions with Ser20 and Gln8 and 8 hydrophobic interactions including Thr19, Ala65, Val35, Arg11, Thr10, Gln34, Gln 9, Cys16; exhibited hydrophobic interactions with Ser556, Phe193, Tyr448, Asn316, Cys366, Leu384, Pro197, Tyr415 and a single hydrophilic interaction with Met414 respectively. 5b interacts best with serotype 3 of DENV having hydrophobic interactions with Leu176, Val212, Tyr200, Gly203, Tyr211, Ser213, Ser185, Asn202, Val86, Gly183, Pro182 and hydrophilic interaction with Gly201.

5. CONCLUSION

There is no fool proof treatment against HCV and DENV as the drug that is effective against one serotype might not inhibit a heterologous serotype. Hence the world is now looking at coming up with a treatment that gives a combinational effect by acting against different sites of the protein's active site. In this study, the enantiomers showed good interaction with the non-structural proteins of HCV and DENV. However, in-vitro studies have to be carried out in order to confirm the pharmacophore property of these lignins.

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BIOSENSOR: A PREDOMINANT CONNECTED SCIENCE INNOVATION

Ligi Milesh[1]**, Ankit Kumar Dubey[1]
[1]REVA University, Department of Biotechnology, School of Applied Sciences, Yelahanka, Bangalore -560064

ABSTRACT: The capacity to survey wellbeing status, illness beginning and movement, and screen treatment resulting through a non-intrusive technique is the principle intend to be accomplished in human services advancement, conveyance, and research.Any existence without the sense is unthinkable. A distinct individual will have five kinds of detecting components, for example nose, tongue, ears, eyes and skin. These detecting components speak to the fundamental sorts of sensors happening in nature.From the sense, there is an advancement of sensors and undoubtly sensors have progressed toward becoming part what's more, bundle of our everyday life. Sensor innovation speaks to one of the developing regions of material science, hardware, besides biotechnologies in the comparative path independently in microelectronics, optical and software engineering advances. Presently multi day's sensors have been considered as an exceptionally potential field of logical research. Nevertheless, biosensors have crucial significance in various biomedical instrumentations, because there is a progressive advancement in the field of biosensors for logical research.A wide scope of methods can be utilized for the improvement of biosensors. Their coupling with high-fondness biomolecules permits the delicate and specific identification of a scope of analytes.

***Keywords*:** Sensor,Biosensors, Analytes, Transducer, biorecognition element, applications.

**** Corresponding author: Dr. Ligi Milesh, PhD ***,** Assistant Professor, REVA University**,** Department of Biotechnology, School of Applied Sciences
Email id: ligimilesh81@gmail.com

1. INTRODUCTION

In late decades, expanded learning about the organic limit of catalysts has made it conceivable to make another age of items and procedures. Among these items are quite biosensors, which speak to an amazing option in contrast to customary investigative strategy [1].As of late, there have been significant innovative progressions in the assembling, types, and applications of biosensors. Applications incorporate clinical and non-clinical diagnostics for home, bio-barrier, bio-remediation, condition, farming, and the nourishment industry. Biosensors have advanced past the discovery of organic dangers for example, *Bacillus anthracis* and are finding use in a number of non-natural applications. Developing biosensor innovations such as lab-on-a-chip have upset the coordination approaches for a very flexible, imaginative, and easy to understand stage.This innovation has progressed impressively lately, fundamentally because of the production of gadgets connected in the territory of biomedicine. These trend-setting innovations have been bit by bit exchanged evenly to different areas, for example, the earth and the agro-sustenance industry. [2]

A biosensor is a gadget that estimates natural or synthetic responses by creating signals relative to the convergence of an analyte in the response. Biosensors are utilized in applications, for example, malady observing, sedate disclosure, and identification of contaminations, illness causing microorganisms and markers that are pointers of an infection in natural liquids (sweat,saliva, blood, urine) [3].Mechanically, a biosensor is a test that incorporates an organic segment, for example, an entire bacterium or an organic item (e.g., a

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protein or counter acting agent) with an electronic part to yield a quantifiable flag. Biosensors, which arrive in a huge assortment of sizes and shapes, are utilized to screen changes in natural conditions. [4]

Biosensors have increased huge consideration lately in drug and nanotechnology, and there is a developing enthusiasm for its application in tissue designing. Since the advancement of the main oxygen biosensor by Lyons and Clark in 1962 [5], analysts in different fields have built up various biosensors for applications in drug, biotechnology, and guard against bioterrorism, just as sustenance, beverages, and natural and rural applications[6].

2. COMPONENTS OF BIOSENSOR

Biosensors are characterized as scientific gadgets comprising of organic atom offering particularity to analyte (it is additionally called biorecognition component) and physico-concoction transducer giving quantifiable flag functioning as a physical sensor [7-8].A biosensor normally comprises of three components; a biorecognition, biotransducer, and an electronic framework that incorporate a processor, flag enhancer, and show [9-10]. Biorecognition framework intercedes particular biocatalysis or explicit official of analyte.Transducers can gauge flag emerging from delivering associations and five gatherings of transducers are commonly known: electrochemical and optical pursued by mass-based are the most widely recognized yet warm and attractive biosensors are notable too[11].

2.1 Biorecognition framework

For acknowledgment of an objective substance in a biosensor, the biomolecule or atomic congregations that have the ability of perceiving the substance is connected. The most generally utilized biomolecules in biosensors are antibodies, layer cuts,proteins, or entire cells..They respond with an analyte to deliver a few biochemical flag, which is identified by the transducer present in a biosensor. Chemicals have been the most generally utilized biomolecules in biosensor applications[12-13]Biorecognition components can be isolated into two gatherings: biocatalytical receptors including chemicals, entire cells, cell organelles, tissues and entire microorganism and bioaffinity receptors including antibodies, cell receptors or nucleic acids.

2.2 Immobilization

Immobilization is either a physical or a concoction technique for capturing the entire biorecognition or making a connection of its part with the transducer surface. Determination of a reasonable immobilization procedure is one of urgent strides of sensor planning in light of the fact that the likelihood of biorecognition component inactivation brought about by picking wrong immobilization technique is high [14] Two kinds of immobilization methods are commonly known - physical and concoction (chemical).Choice of progressively suitable technique relies upon nature of the picked biorecognition component, utilized transducer, physico-concoction conditions and properties of analyte. [15]

2.3 Transducer

A transducer is a gadget that changes over an organic flag gotten from a biochemical response between the organic segment and an analyte into an electronic flag and passes it to the indicator framework. The transducer acts like an interpreter, changing biochemical flag into another flag to be translated by the processor. Transducer might be electrochemical, optical, warm or piezoelectric relying on the kind of flag got. The biochemical transducer or biocomponent gives the biosensor the selectivity or explicitness. The transducer of a gadget reacts so that a flag can be electronically intensified and showed. The physical transducers change from electrochemical, spectroscopic, warm, piezoelectric and surface acoustic wave innovation [6][16].

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3. CHARACTERISTICS OF A BIOSENSOR

There are sure static and dynamic qualities that each biosensor has. The improvement of these properties is pondered the execution of the biosensor [6]. Some important properties of biosensors are:

Selectivity: - The capacity of a bioreceptor to identify an explicit analyte in an example containing different admixtures and contaminants. The association of an antigen with the neutralizer delineates the best case of selectivity.

Reproducibility: - The capacity of the biosensor to create indistinguishable reactions for a copied test set-up. The reproducibility is portrayed by the exactness and precision of the transducer and hardware in a biosensor.

Soundness:-The level of powerlessness to encompassing unsettling influences in and around the biosensing framework. These unsettling influences can cause a float in the yield signs of a biosensor under estimation. This can cause a blunder in the deliberate fixation and can influence the exactness and precision of the biosensor. Solidness is the most critical element in applications where a biosensor requires long hatching advances or ceaseless checking.

Sensitivity: -The base measure of analyte that can be identified by a biosensor characterizes its limit of discovery (LOD) or affectability. In various restorative and natural checking applications, a biosensor is required to distinguish analyte convergence of as low as ng/ml or even fg/ml to confirmthe nearness of hints of analytes in an example.

Linearity: -Linearity of the biosensor can be related with the goals of the biosensor and scope of analyte fixations under test. The goals of the biosensor is characterized as the littlest change in the grouping of an analyte that is required to acquire a change the reaction of the biosensor [6].

4. PRINCIPLE AND WORKING OF A BIOSENSOR

The ideal natural material (for the most part a particular protein) is immobilized by regular techniques (physical or film ensnarement, non-covalent or covalent authoritative). This immobilized natural material is in private contact with the transducer. The analyte ties to the natural material to frame a bound analyte, which thus produces the electronic reaction that can be estimated. In a few cases, the analyte is selected based on the item, which might be related with the arrival of warmth, gas (oxygen), electrons or hydrogen particles. The transducer can change over the thing-associated changes into electrical signs, which can be heightened and evaluated [17].

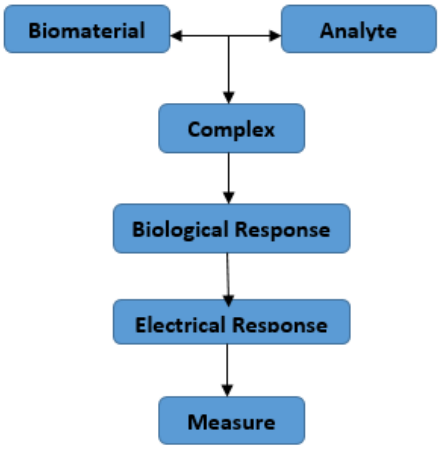

Fig 1: General measurement flow for a biosensor [17].

The electrical flag from the transducer is regularly low and superimposed upon a moderately high and boisterous (for example containing a high recurrence flag segment of a clearly

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irregular nature, because of electrical obstruction or created inside the electronic parts of the transducer) pattern. The flag handling regularly includes subtracting a 'REFERENCES' benchmark flag, got from a comparable transducer with no biocatalyst layer, from the example flag, enhancing the resultant flag distinction and electronically separating (smoothing) out the undesirable flag commotion. The generally moderate nature of the biosensor reaction impressively facilitates the issue of electrical clamour filtration. The simple flag delivered at this stage might be yield straightforwardly however is generally changed over to an advanced flag and go to a chip arrange where the information is handled, controlled to wanted units and yield to a showcase gadget or information store.

5. TYPES OF BIOSENSORS

Biosensors are gadgets and these are utilized in material science, science and science. Biosensors are utilized to dissect different physical, substance and organic procedures. Warmth, light and power are the consequences of different responses. Those properties are abused to dissect responses. There are distinctive sorts of biosensors relying upon the sort of property used to break down a response.

Their common part or their transduction segment can accumulate biosensors. Common segments consolidate synthetic substances, antibodies, regular tissue, organellesand scale down scale animals. The strategy for transduction depends upon the kind of physicochemical change coming about because of the distinguishing event. Biosensors subject to transducer segment are mass based (piezoelectric, etc), electrochemical biosensors (potentiometric, amperometric, etc), and optical sorts of biosensors (fiber optics, etc)[18].

Piezoelectric Sensors: In this mode, recognizing molecules are annexed to a piezoelectric surface - a mass to repeat transducer - in which joint efforts between the analyte and the identifying particles set up mechanical vibrations that can be changed over into an electrical banner comparing to the proportion of the analyte.

Electrochemical Sensors: In this system, quantifiable electrons or particles are delivered or smothered by various kinds of concoction responses. The recognizing particles react expressly with blends to be recognized, beginning an electrical banner in respect to the combination of the analyte. In context of their working guideline, the electrochemical biosensors can utilize potentiometric, amperometric and impedimetric transducers changing over the substance data into a quantifiable amperometric flag.

Optical Sensors: In this mode, the optical strands permit discovery of analytes based on ingestion, fluorescence or light dissipating. The reaction causes an alteration in fluorescence or absorbance coming about because of advancement in the refractive record of the surface between two media, which differentiate in thickness [17].

6. APPLICATIONS OF BIOSENSORS

6.1 Medical Diagnostics: Lately, various sensors dependent on metabolites are accessible for observing blood glucose, lactate, cholesterol, urea, uric corrosive and so forth, which are clinically vital. These biosensors offer a preferred standpoint to research centre investigation of important substances for clinical examination [19].Biosensors can be of colossal significance in tissue designing applications, especially in keeping up three dimensional cell societies [20-21] and creating "organs-on-chips" models

6.2 Food and Agriculture: The expanding shopper requests for quality and safe sustenance requires a ton of endeavours for quality control by the industries. Ordinary strategies are arduous, time expending and costly. As of late, proficient, quick and exact scientific strategies have been produced utilizing biosensors that empower on-line estimations amid the bioprocesses [22].Their execution in-line is constrained by the need of sterility, visit

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alignment, analyte weakening, and so on. Potential uses of compound based biosensors to sustenance quality control incorporate estimation of proteins, starches, vitamins, inorganic particles, sugar content etc. [23].

6.3 Biodefense applications: The essential aim of such biosensors is be to gently and explicitly perceive living things introducing peril in every practical sense persistent called biowarfare administrators (BWAs) to be explicit, tiny living beings (vegetative and spores), harms and infections. A few undertakings to contraption such biosensors has been done using nuclear techniques which can see the substance markers of BWAs [24]

6.4 Fiber optic: The utilization of an optical fiber as biosensors are making progress because of a few of its highlights like the capacity to lead light and resistance to electrical and attractive impedance. A normal optical biosensor utilizes absorbance estimations to decide an adjustment in centralization of analytes that assimilate a specific wavelength of light. The light is transmitted through an optical fiber to the example; the measure of light consumed by the analyte is distinguished through a similar fiber or a second fiber. The organic material is immobilized toward the finish of the optical fiber. The principle preferred standpoint of optical biosensors is its low cost [17].

6.5 Environmental Monitoring: Ecological water checking is a region in which entire cell biosensors may have generous focal points for fighting the expanding number of toxins finding their way into the groundwater frameworks and henceforth into drinking water. Essential focuses for contamination biosensors now incorporate anionic poisons, for example, nitrates and phosphates[17].

6.6 Industry: Alongside ordinary modern maturation creating materials, numerous new items are being delivered by substantial scale bacterial and eukaryotes cell culture. The observing of these sensitive and costly procedures is basic for limiting the expenses of creation; explicit biosensors can be intended to quantify the age of an aging item[25]

7. CONCLUSION

A few recently created methods extending from electrochemical, optical including fluorescence-based, and electromechanical are current transducing techniques, which are employed in the advancement of biosensors.Its fundamental favourable position is that it compasses to everyone's hand for different valuable applications, because of its minimal effort, straightforwardness, substantial speed and exactness. In view of different transduction advances, the greater part of the examination is centered onenhancing affectability, selectivity, and security. Most business biosensors created until date is expected to center in clinical applications. Anyway, different applications zones like nourishment, pharmaceutical, agribusiness, and condition are still to be investigated.

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PEPTIDOMIMETICS: STRUCTURAL AND PHARMACOLOGICAL IDENTIFICATION OF NOVEL PEPTIDE OF TARGET CHYMASE ENZYME ACTIVITY TO PREDICT ASTHMA TREATMENT

Nisha Rajashekar[1], Asiya Rozinder[2], D.B.M.Virupakshaiah[2], Prashantha CN[1]
[1]Department of Biotechnology, REVA University, Bangalore-560092
[2]Department of Biotechnology, Basaveshwar Engineering College, Bagalkot

ABSTRACT: Chymase enzyme is an important target for the treatment of Asthma and allergic diseases. It primarily presents in mast cells that have the role of angiotensin II, an angiotensin-converting enzyme and is an important functional class of many allergic, inflammatory and heart disease. The pathophysiology and molecular function of chymase enzyme are unknown. Studying various literatures to find the targeted function of chymase and used for treatment of diseases. Using bioinformatics methods to identify disease target protein, protein functions and peptide design that helps to mimic the protein-peptide interaction to inhibit chymase activity. We selected chymase enzyme sequence from uniprot database and this sequence is used to predict physicochemical properties, protein structure prediction and peptide design. Here, we predicted 95 clusters of peptide sequences of which all peptides were used for pharmacophore and protein-protein docking. The molecular docking of the novel peptide with chymase protein has predicted and virtually screened based on RMSD values. The predicted novel peptides of chymase protein with strong putative inhibitor of asthma and allergic diseases that can enhance the alveolar cells in lungs. Furthermore, a series of peptidomimetic molecules were designed from novel peptides and screened for their inhibitory interactions which ultimately led to a small set of peptidomimetic inhibitors of chymase enzyme. These peptidomimetic compounds can be used as novel therapeutic functions of asthma.

Keywords Chymase, Asthma, Peptide, Peptidomimetics, molecular docking, allergic diseases

Corresponding author: Dr.C.N.Prashantha
Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore, India
Email: Prashantha.cn@reva.edu.in

1. INTRODUCTION

Asthma is a chronic respiratory disease affected by air pollution and other genetic factors [1]. There is about 60% of asthma patients is affected because of air pollution and 10% of the population is in genetic change [2,3]. Asthma characterized by shortness of breath, wheezing, recurring cough and excess mucus production in the airway smooth muscle cells [4]. It integrates known and unknown target cells that show a multicellular mechanism to cause the disease. There is an abnormal response of different cell types such as immune cells, biochemical molecules, and molecular genes and proteins mainly trigger in asthma and other allergic diseases [5-7]. The epithelium of immune cells is the primary response to cause disease; there are cytokine response interleukin (IL)-3, IL-4, IL-13 and IL-9 that develops in allergic pathogenesis [8, 9]. The epithelial cells also produce mast cells and innate lymphoid cells that also triggered in asthma and other allergic diseases [10, 11].
Mast cells store and generate a large number of substances such as serine proteases, histamines, interleukins (IL-1, IL6), tumor necrosis factor–α (TNF-α), platelet-derived

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growth factor (PDGF) and transforming growth factor-β (TGFβ). The mast cell substances directly attached with IgE immunoglobulin to the cell membrane of the alveolar region of lungs [12, 13]. Mast cells contain two types of cells such as serine protease tryptase, a trypsin-like enzyme, and chymase, a chymotrypsin-like enzyme. The chymase activity specifically within smooth muscle tissue within airway passage that significantly affects lung functions [14]. Mast cells have less complex structure contains protease group of predominant enzymes [15, 16] that has positive cell attachment with the airway smooth muscle cells that contribute better lung function [17]. Based on different reviews and literature have shown less known functions of chymase enzyme activity within asthma. Although there is a need completely rule out complete possibilities of understanding the function of chymase activity within asthma and allergic diseases [18-20].

In the present research targeted two major objectives such as understand the mechanism of targeted chymase enzyme function and develop the novel antiasthma drug molecule that targets chymase enzyme to control the disease progression. Recent advances in the techniques of synthetic peptide and peptidomimetic small molecules have increased the potential for the creation of specific inhibitors of immune response chymase protein processes in asthma and allergic diseases. The peptides and peptidomimetic organic small molecules that mimic the action of peptides. The peptide has structurally resembled their molecular backbone and the interactions with proteins can be mediated by other molecules. Using computational approaches to design the peptide and also to study the stereochemical activity of the peptide binds to target chymase enzyme that can predict drug-like property.

2. MATERIALS AND METHODS

2.1. Protein sequence selection

Mast cell secretary substances with a suspected role in vasoactive peptide generation, extracellular matrix degradation, and regulation of gland secretion sequence were downloaded from UniProt (http://www.uniprot.org/uniprot/P23946) was the sequence for this study [21]. We have studied protein sequence with physic-chemical properties and predicting secondary structure and hierarchical approach of 3D structure and function prediction followed by an ab-initio method using I-TASSER [22]. The resulting 3D structure and functional characters were further used for peptidomimetic study.

2.2. Identification of the peptide, peptide modeling, and target screening

Chymase enzyme sequence was used to find the peptide using PeptideMass [23, 24]. PeptideMass cleaves protein sequence with default enzyme and computes the mass of each generated peptide. The resulting theoretical isoelectric point and mass values were further to carry out a post-translational study to predict peptidomimetic properties. The resultant peptide sequences were carried out using PEP-FOLD server (http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/) [25], a de novo method of predicting peptide 3D structures between 3-50 amino acids. It uses Hidden Markov Model that characterizes protein backbone conformation and series of overlapping fragments of four residue lengths. It determines structural alphabet of the sequence to build a model by assembly using a greedy procedure driven by a coarse-grained energy score [26, 27]. It generates clusters of models ranked on the basis of Optimized Potential for Efficient structure Prediction Energy (OPEP). Top-ranked 10 peptide models were screened based on decreasing order of OPRP scores.

2.3. Molecular Docking

To explore the target binding potential protein structure is selected from Protein databank (PDB) (http://www.rcsb.org/pdb) [28]. Further have predicted 3D protein structure with stereochemical properties of peptide bonds can predict using SAVS [29]. The SAVS has five

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different parameters such as PROCHECK, WHAT_CHECK, ERRAT, VERIFY_3D and PROVE to understand the stereochemical quality of protein structure by residue-by-residue geometry along with calculated macromolecular Z-score deviation of the model with the high-quality resolution of protein structures. CastP calculation server is used to predict ligand binding sites employed to calculate surface pockets of the target receptor [29]. Protein-peptide docking study was carried out using PatchDock [30]. The docking complexes were visualized using Discovery studio 4.0 software suite. The docking properties of protein-peptide interaction along with molecular properties were predicted for the lead peptides. The possible strong binding interactions were mimics the functional sites of protein modification were predicted as best target peptidomimetic drug.

2.4. Structure-based peptidomimetics design

The peptidomimetic properties of top-ranked peptides were using SuperMimic software [31]. This software identifies compounds that mimic parts of a protein, or positions in proteins that are suitable for inserting mimetics. We selected top-ranked 10 peptide structure that contains peptidomimetic building blocks on the one hand and protein structures on the other. The search for promising peptidomimetic linkers for a given peptide is based on the superposition of the peptide with several conformers of the mimetic. The screening for spacer at a given position in template peptide is based on the spatial superposition of four stem atoms of the template (N and Cα atom of the first residue and Cα and C atom of the last residue) with the analogous atoms of the suitable mimetic peptide. From amongst the screened spacers/mimetics obtained for specific positions of the top-ranked peptide, those having least RMSD of their main chain atoms from that of template peptide were further selected. These selected spacers were inserted at a respective position in a top-ranked peptide by replacing the corresponding residue followed by capping peptide termini using Discovery Studio 4.0. The screening library comprises of peptidomimetic building blocks from the literature such as D-amino acids, α-helices, β-strand, β-turn, γ-turn and peptide mimetic extracted from PDB crystal structure complexes.

3. RESULTS AND DISCUSSION

In this study, we have used chymase protein sequence that used to study physic-chemical properties and predict secondary structure. The chymase has a molecular weight of 27324.80 and theoretical pI of 9.44 instability index (II) is computed to be 41.20 and the classified protein as unstable (table: 1).

Table: 1 Physico-chemical properties of Chymase protein

Rank	PDB Hit	TM-score	RMSDa	IDENa	Cov
1	1klt_A	0.905	0.64	0.996	0.915
2	2cp1_	0.899	0.68	0.507	0.911
3	2rdl_A	0.898	0.9	0.708	0.915
4	1fi8_B	0.897	0.79	0.52	0.911
5	1iau_A	0.894	0.75	0.531	0.907
6	1euf_A	0.893	0.64	0.552	0.903
7	3rp2_B	0.891	0.71	0.596	0.903
8	3tju_A	0.889	0.74	0.493	0.903
9	3fzz_A	0.884	0.99	0.496	0.907
10	1cgh_A	0.882	1.16	0.511	0.903

I-TASSER also have biological annotation target program such as COFACTOR and COACH

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to predict active site identification that comprises to predict protein-protein interaction (Figure: 1). B-factor profile in the figure below corresponds to the normalized B-factor of the target protein, defined by B= (B'-u)/s, where B' is the raw B-factor value, u and s are respectively the mean and standard deviation of the raw B-factors along the sequence (table:2).

Table: 2. biologically active target protein active sites prediction using COFACTOR tool from I—TASSER.

Rank	C score EC Hit	PDB	TM-score	RMSDa	IDENa	Cov	EC Number	Active Site Residues
1	0.652	1pjpA	0.901	0.81	0.987	0.915	3.4.21.39	33, 35, 125, 127, 143, 160, 162, 201, 204, 207
2	0.63	1kltA	0.905	0.64	0.996	0.915	3.4.21.39	63, 107, 195, 197, 198
3	0.546	1eufA	0.893	0.64	0.552	0.903	3.4.21.-	63, 107, 196, 199, 213
4	0.526	1kynB	0.881	1.24	0.513	0.907	3.4.21.20	64, 199, 200
5	0.517	1avwA	0.85	1.27	0.385	0.883	3.4.21.4	66, 110, 201, 202

The resultant protein structure is used to predict peptides using the PeptideMass tool. The PeptideMass has default parameters such as selected enzyme trypsin that shows the theoretical peptide masses and position of the peptide present. The sequence annotations for the protein in order to generate the peptide mass (table: 3).

Table: 3. Identification of peptide sequences from target protein using PeptideMass tool

Mass	Position	Peptide sequence
3505.7089	17-49	AEAGEIIGGTECKPHSRPYM AYLEIVTSNGPSK
2416.3023	120-142	ASLTLAVGTLPFPSQFNFVP PGR
2272.1091	71-90	SITVTLGAHNITEEEDTWQK
1976.9858	201-221	GDSGGPLLCAGVAQGIVSYG R
1855.1311	16-31	MLLLPLPLLLFLLCSR
1852.9816	233-247	ISHYRPWINQILQAN
1685.8679	102-115	YNTSTLHHDIMLLK
1627.7645	180-193	DFDHNLQLCVGNPR
1571.8639	152-166	TGVLKPGSDTLQEVK
1304.5874	169-179	LMDPQACSHFR
1259.6313	59-70	NFVLTAAHCAGR
1188.6371	222-232	SDAKPPAVFTR
912.476	50-57	FCGGFLIR
645.3467	146-151	VAGWGR
601.3919	91-95	LEVIK

The protein sequence has a biologically active region at 22-245 peptidases S1 activity, which contains sequence conflicts at 28cys-Ser and 131-132 FP- AV AA sequences. The sequence

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also contains natural variants at 46Gly-Arg, 66His-Arg, and 98 Arg-His corresponding variants, there is a chemical binding region at Ser197, Ser218 and Gly220 were used as potential targets for substrate binding. Based on this biological inactive property of sequence, we have selected the peptide sequence with inactive functional region shows GDSGGPLLCAGVAQGIVSYGR peptide sequence is used for peptidomimetic properties. The PEP-FOLD served predicts de novo peptide structure from given peptide sequence of length 3-25 amino acids. It generates best models from the generated clusters. The clustering scores of top-ranked peptide sequences were selected based on OPEP energies of their represented models. We got 95 clusters of models generated and top 5 models (table: 4). The molecular dynamic simulation of 22 amino acids in model protein structures with RMSD plot obtained along with trajectory of the simulation showed relatively stable region 17ns onwards. Further, the comparative structural analysis (in PyMol) of peptide conformations extracted along the stable region of the trajectory with the best-predicted model from PEP-FOLD showed RMSD of ~0.5 Å. Thus this evaluation indicates the possible relative structural stability of predicted top ranked 5 peptide models.

Table: 4. PEP-FOLD clusters prediction to build peptide structure using novel peptide sequence.

# Clusters	file	sOPEP	avg	gdt	max	q	tm
# cluster 1 - Size: 1	PEPFOLD-model1	-46.5649	0.262	0.349	0.252	0.232	0.213
# cluster 2 - Size: 1	PEPFOLD-model2	-45.3969	0.268	0.36	0.262	0.238	0.211
# cluster 3 - Size: 2	PEPFOLD-model3	-44.2642	0.294	0.392	0.295	0.248	0.24
	PEPFOLD-model3.1	-39.86	0.274	0.369	0.27	0.232	0.224
# cluster 4 - Size: 1	PEPFOLD-model4	-43.1607	0.288	0.382	0.284	0.248	0.237
# cluster 5 - Size: 1	PEPFOLD-model5	-43.0051	0.298	0.391	0.3	0.254	0.247
# cluster 6 - Size: 1	PEPFOLD-model6	-42.9005	0.269	0.354	0.267	0.234	0.223
# cluster 7 - Size: 1	PEPFOLD-model7	-41.7889	0.275	0.366	0.273	0.238	0.225
# cluster 8 - Size: 1	PEPFOLD-model8	-41.658	0.279	0.372	0.276	0.239	0.229
# cluster 9 - Size: 1	PEPFOLD-model9	-41.4571	0.283	0.372	0.284	0.242	0.233
# cluster 10 - Size: 1	PEPFOLD-model10	-41.4532	0.287	0.377	0.287	0.249	0.235
# cluster 11 - Size: 1	PEPFOLD-model11	-41.2482	0.289	0.381	0.293	0.243	0.238
# cluster 12 - Size: 1	PEPFOLD-model12	-41.024	0.288	0.38	0.287	0.244	0.239
# cluster 13 - Size: 1	PEPFOLD-model13	-40.9376	0.288	0.38	0.291	0.243	0.237
# cluster 14 - Size: 1	PEPFOLD-model14	-40.6044	0.272	0.361	0.265	0.243	0.217

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# cluster 15 - Size: 1	PEPFOLD-model15	-40.5418	0.268	0.354	0.263	0.238	0.217
# cluster 16 - Size: 1	PEPFOLD-model16	-40.5245	0.277	0.369	0.273	0.241	0.224
# cluster 17 - Size: 1	PEPFOLD-model17	-40.4257	0.291	0.382	0.293	0.252	0.236
# cluster 18 - Size: 1	PEPFOLD-model18	-40.4003	0.281	0.375	0.275	0.245	0.228
# cluster 19 - Size: 1	PEPFOLD-model19	-40.375	0.278	0.362	0.279	0.244	0.226
# cluster 20 - Size: 1	PEPFOLD-model20	-40.2384	0.299	0.394	0.297	0.26	0.245
# cluster 21 - Size: 1	PEPFOLD-model21	-40.2334	0.293	0.389	0.291	0.251	0.241
# cluster 22 - Size: 1	PEPFOLD-model22	-40.0875	0.271	0.363	0.264	0.236	0.221
# cluster 23 - Size: 1	PEPFOLD-model23	-40.0365	0.286	0.378	0.284	0.25	0.233
# cluster 24 - Size: 1	PEPFOLD-model24	-39.7633	0.291	0.385	0.291	0.246	0.24
# cluster 25 - Size: 1	PEPFOLD-model25	-39.6443	0.278	0.371	0.277	0.236	0.228
# cluster 26 - Size: 1	PEPFOLD-model26	-39.4577	0.272	0.356	0.274	0.232	0.225
# cluster 27 - Size: 1	PEPFOLD-model27	-39.3775	0.282	0.369	0.28	0.247	0.233
# cluster 28 - Size: 1	PEPFOLD-model28	-39.1021	0.275	0.368	0.272	0.236	0.224
# cluster 29 - Size: 1	PEPFOLD-model29	-38.8088	0.265	0.346	0.263	0.231	0.22
# cluster 30 - Size: 1	PEPFOLD-model30	-38.702	0.288	0.38	0.287	0.252	0.234
# cluster 31 - Size: 1	PEPFOLD-model31	-38.6362	0.277	0.365	0.281	0.238	0.226
# cluster 32 - Size: 1	PEPFOLD-model32	-38.4381	0.287	0.378	0.287	0.247	0.236
# cluster 33 - Size: 2	PEPFOLD-model33	-38.2888	0.304	0.392	0.311	0.258	0.254
	PEPFOLD-model33.1	-34.0703	0.293	0.383	0.297	0.25	0.242
# cluster 34 - Size: 1	PEPFOLD-model34	-38.2778	0.275	0.364	0.27	0.245	0.223
# cluster 35 - Size: 1	PEPFOLD-model35	-38.0893	0.292	0.386	0.294	0.249	0.241
# cluster 36 - Size: 1	PEPFOLD-model36	-38.0832	0.281	0.368	0.282	0.242	0.232
# cluster 37 - Size: 1	PEPFOLD-model37	-37.9823	0.283	0.371	0.282	0.251	0.227

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# cluster 38 - Size: 1	PEPFOLD-model38	-37.7844	0.272	0.354	0.268	0.237	0.227
# cluster 39 - Size: 1	PEPFOLD-model39	-37.5409	0.273	0.37	0.269	0.236	0.218
# cluster 40 - Size: 1	PEPFOLD-model40	-37.4656	0.284	0.377	0.281	0.251	0.228
# cluster 41 - Size: 1	PEPFOLD-model41	-37.2988	0.281	0.374	0.279	0.242	0.23
# cluster 42 - Size: 1	PEPFOLD-model42	-37.2722	0.279	0.369	0.279	0.241	0.229
# cluster 43 - Size: 1	PEPFOLD-model43	-37.2461	0.261	0.349	0.255	0.232	0.208
# cluster 44 - Size: 1	PEPFOLD-model44	-37.2189	0.289	0.381	0.294	0.244	0.237
# cluster 45 - Size: 1	PEPFOLD-model45	-36.9127	0.283	0.38	0.283	0.24	0.23
# cluster 46 - Size: 1	PEPFOLD-model46	-36.8666	0.303	0.397	0.307	0.26	0.249
# cluster 47 - Size: 1	PEPFOLD-model47	-36.7345	0.282	0.375	0.285	0.239	0.229
# cluster 48 - Size: 1	PEPFOLD-model48	-36.6492	0.285	0.374	0.285	0.247	0.233
# cluster 49 - Size: 1	PEPFOLD-model49	-36.5817	0.292	0.382	0.292	0.254	0.239
# cluster 50 - Size: 2	PEPFOLD-model50	-36.5577	0.29	0.386	0.288	0.251	0.235
	PEPFOLD-model50.1	-36.1701	0.281	0.371	0.279	0.243	0.229
# cluster 51 - Size: 1	PEPFOLD-model51	-36.5345	0.285	0.373	0.294	0.237	0.238
# cluster 52 - Size: 1	PEPFOLD-model52	-36.4376	0.266	0.355	0.259	0.234	0.217
# cluster 53 - Size: 1	PEPFOLD-model53	-36.3727	0.274	0.367	0.273	0.231	0.223
# cluster 54 - Size: 1	PEPFOLD-model54	-36.2731	0.263	0.353	0.259	0.229	0.211
# cluster 55 - Size: 1	PEPFOLD-model55	-36.0977	0.286	0.375	0.29	0.241	0.239
# cluster 56 - Size: 2	PEPFOLD-model56	-36.0574	0.297	0.395	0.3	0.25	0.242
	PEPFOLD-model56.1	-34.293	0.302	0.402	0.304	0.254	0.247
# cluster 57 - Size: 1	PEPFOLD-model57	-36.0443	0.28	0.372	0.278	0.24	0.229
# cluster 58 - Size: 1	PEPFOLD-model58	-36.0245	0.279	0.368	0.282	0.24	0.228
# cluster 59 - Size: 1	PEPFOLD-model59	-35.9391	0.278	0.373	0.276	0.24	0.224

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# cluster 60 - Size: 1	PEPFOLD-model60	-35.935	0.245	0.324	0.246	0.215	0.196
# cluster 61 - Size: 1	PEPFOLD-model61	-35.8919	0.276	0.365	0.271	0.239	0.227
# cluster 62 - Size: 1	PEPFOLD-model62	-35.8659	0.28	0.37	0.282	0.234	0.234
# cluster 63 - Size: 1	PEPFOLD-model63	-35.8527	0.283	0.376	0.284	0.244	0.229
# cluster 64 - Size: 1	PEPFOLD-model64	-35.5598	0.287	0.378	0.287	0.248	0.236
# cluster 65 - Size: 1	PEPFOLD-model65	-35.5547	0.274	0.362	0.275	0.238	0.221
# cluster 66 - Size: 1	PEPFOLD-model66	-35.5521	0.269	0.36	0.264	0.236	0.216
# cluster 67 - Size: 1	PEPFOLD-model67	-35.5035	0.301	0.398	0.303	0.259	0.243
# cluster 68 - Size: 1	PEPFOLD-model68	-35.2365	0.287	0.382	0.284	0.245	0.236
# cluster 69 - Size: 1	PEPFOLD-model69	-35.1283	0.267	0.357	0.26	0.239	0.212
# cluster 70 - Size: 1	PEPFOLD-model70	-35.1128	0.292	0.381	0.297	0.249	0.243
# cluster 71 - Size: 1	PEPFOLD-model71	-35.0828	0.29	0.384	0.291	0.248	0.237
# cluster 72 - Size: 1	PEPFOLD-model72	-34.9331	0.297	0.388	0.304	0.252	0.245
# cluster 73 - Size: 1	PEPFOLD-model73	-34.8083	0.289	0.381	0.29	0.254	0.233
# cluster 74 - Size: 1	PEPFOLD-model74	-34.7893	0.287	0.383	0.293	0.238	0.235
# cluster 75 - Size: 1	PEPFOLD-model75	-34.569	0.291	0.386	0.29	0.252	0.237
# cluster 76 - Size: 1	PEPFOLD-model76	-34.3423	0.291	0.382	0.295	0.252	0.235
# cluster 77 - Size: 1	PEPFOLD-model77	-34.3364	0.298	0.39	0.298	0.258	0.243
# cluster 78 - Size: 1	PEPFOLD-model78	-34.2979	0.288	0.382	0.286	0.25	0.236
# cluster 79 - Size: 1	PEPFOLD-model79	-34.2181	0.277	0.368	0.274	0.238	0.227
# cluster 80 - Size: 1	PEPFOLD-model80	-34.2041	0.278	0.368	0.278	0.239	0.228
# cluster 81 - Size: 1	PEPFOLD-model81	-34.1784	0.287	0.38	0.29	0.247	0.232
# cluster 82 - Size: 1	PEPFOLD-model82	-33.9702	0.274	0.365	0.273	0.236	0.222
# cluster 83 - Size: 1	PEPFOLD-model83	-33.9552	0.288	0.385	0.287	0.25	0.23

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# cluster 84 - Size: 1	PEPFOLD-model84	-33.2191	0.281	0.372	0.284	0.239	0.228
# cluster 85 - Size: 1	PEPFOLD-model85	-32.8554	0.274	0.366	0.269	0.235	0.224
# cluster 86 - Size: 1	PEPFOLD-model86	-32.7997	0.278	0.367	0.282	0.233	0.231
# cluster 87 - Size: 1	PEPFOLD-model87	-32.3347	0.293	0.388	0.294	0.252	0.238
# cluster 88 - Size: 1	PEPFOLD-model88	-31.7692	0.264	0.352	0.259	0.234	0.209
# cluster 89 - Size: 1	PEPFOLD-model89	-31.6663	0.263	0.348	0.258	0.232	0.217
# cluster 90 - Size: 1	PEPFOLD-model90	-31.4226	0.28	0.37	0.282	0.238	0.23
# cluster 91 - Size: 1	PEPFOLD-model91	-31.3433	0.293	0.383	0.296	0.253	0.24
# cluster 92 - Size: 1	PEPFOLD-model92	-31.2836	0.258	0.345	0.257	0.216	0.213
# cluster 93 - Size: 1	PEPFOLD-model93	-31.2104	0.291	0.388	0.291	0.25	0.237
# cluster 94 - Size: 1	PEPFOLD-model94	-31.1437	0.281	0.37	0.285	0.236	0.233
# cluster 95 - Size: 1	PEPFOLD-model95	-30.9497	0.286	0.378	0.287	0.246	0.234
# cluster 96 - Size: 1	PEPFOLD-model96	-30.8113	0.287	0.377	0.288	0.247	0.235

3.1. Molecular docking of protein-peptide interaction

The docking interactions of the target protein with novel peptide structures can be predicted based on interaction. The interaction energy can be calculated based on a cluster of interactions. The novel peptide docked with target protein within interaction energy shows in the table: 6. the score of each interaction that shows strong interaction with peptide structure with a binding energy of -126.2kcal/mol. The peptide sequence from 164-214 amino acids fragment is strongly interacting with the novel designed peptide. The stability of complex docked structure was predicted based on RMSD during simulation was calculated with respect to their initial docking structures (Table: 5, 6) (Figures: Docking_1A, 1B, Docking_clusters_1_10, Cluster_1_2, Clusters overall)

Table: 5. Docking interaction of novel peptide with chymase protein interactions.

Rank	1	2	3	4	5	6	7	8	9	10
Docking Score	-208.02	-197.93	-197.44	-195.59	-194.19	-189.41	-189.35	-186.45	-185.55	-183.97
Ligand rmsd (Å)	39.11	42.29	65	61.59	64.62	63.3	34.22	31.33	43.01	63.97

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Table: 6 Docking poses of top 10 best docked protein peptide interactions with different poses (1- 10).

Cluster name	Docking Score	Ligand rmsd (Å)	Cluster Density	Average (RMSD)	Max RMSD	No of Elements
Cluster_1	-208.02	39.11	20.9944	4.81081	14.4734	101
Cluster_2	-197.93	42.29	19.9454	8.72381	22.7283	174
Cluster_3	-197.44	65.00	18.1631	6.99222	42.5102	127
Cluster_4	-195.59	61.59	16.4343	8.03196	26.8584	132
Cluster_5	-194.19	64.62	11.6139	11.7962	24.8126	137
Cluster_6	-189.41	63.30	9.28039	9.26685	24.2711	86
Cluster_7	-189.35	34.22	7.17117	11.1367	27.2528	91
Cluster_8	-186.45	31.33	6.33338	9.78941	23.3482	62
Cluster_9	-185.55	43.01	5.19451	10.3956	20.5916	54
Cluster_10	-183.97	63.97	5.09667	7.06343	11.9528	36

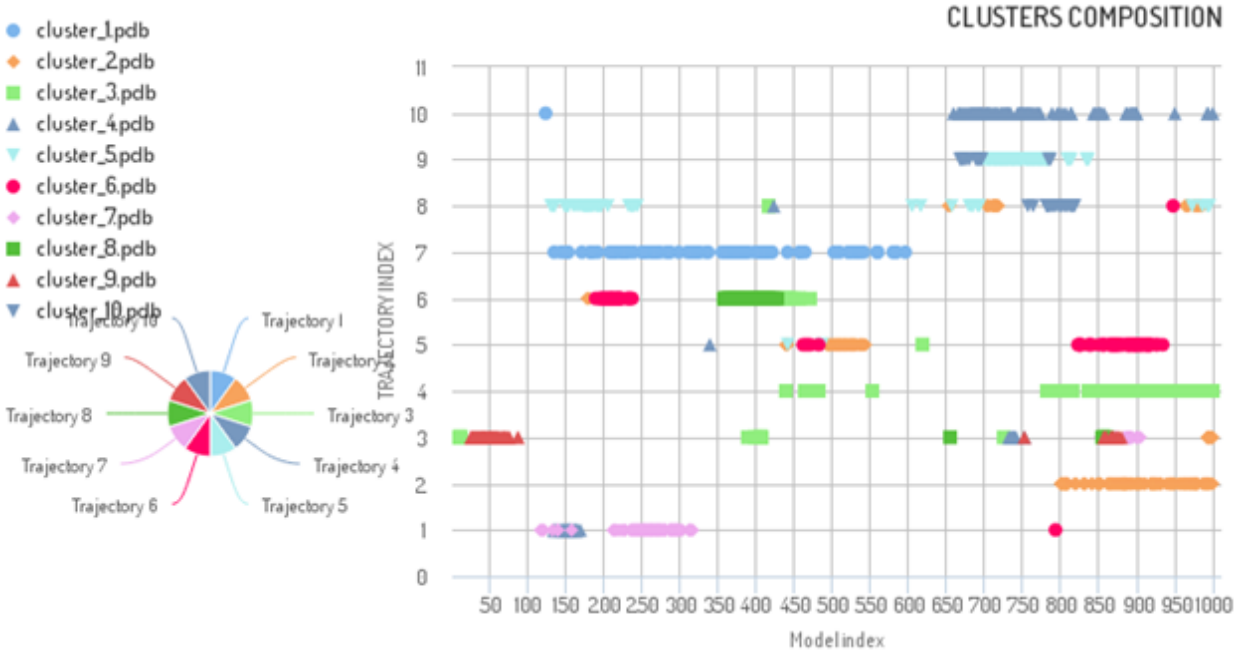

Figure: 1Cluster composition of docking poses of top 10 best docked protein-peptide interactions.

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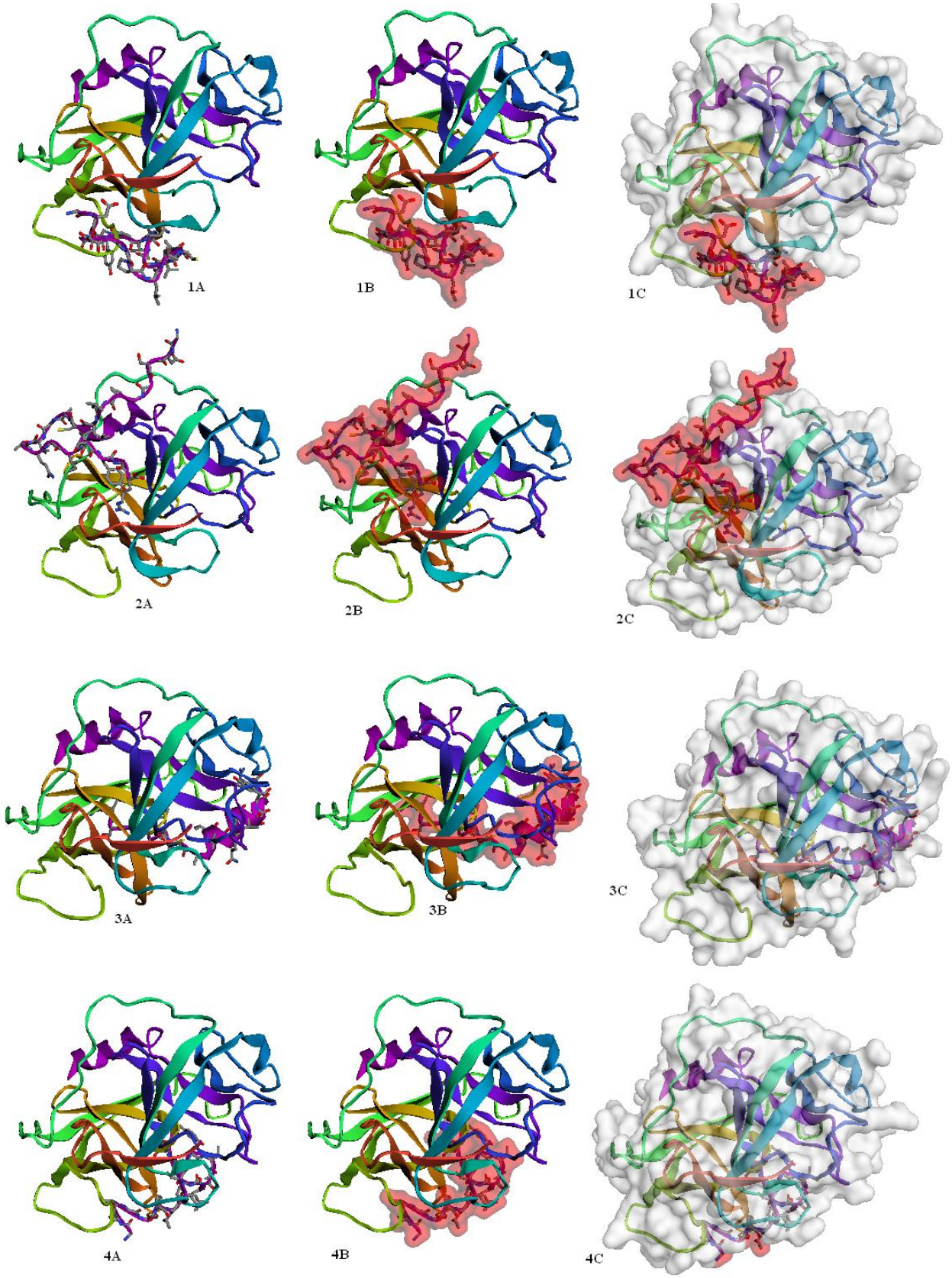

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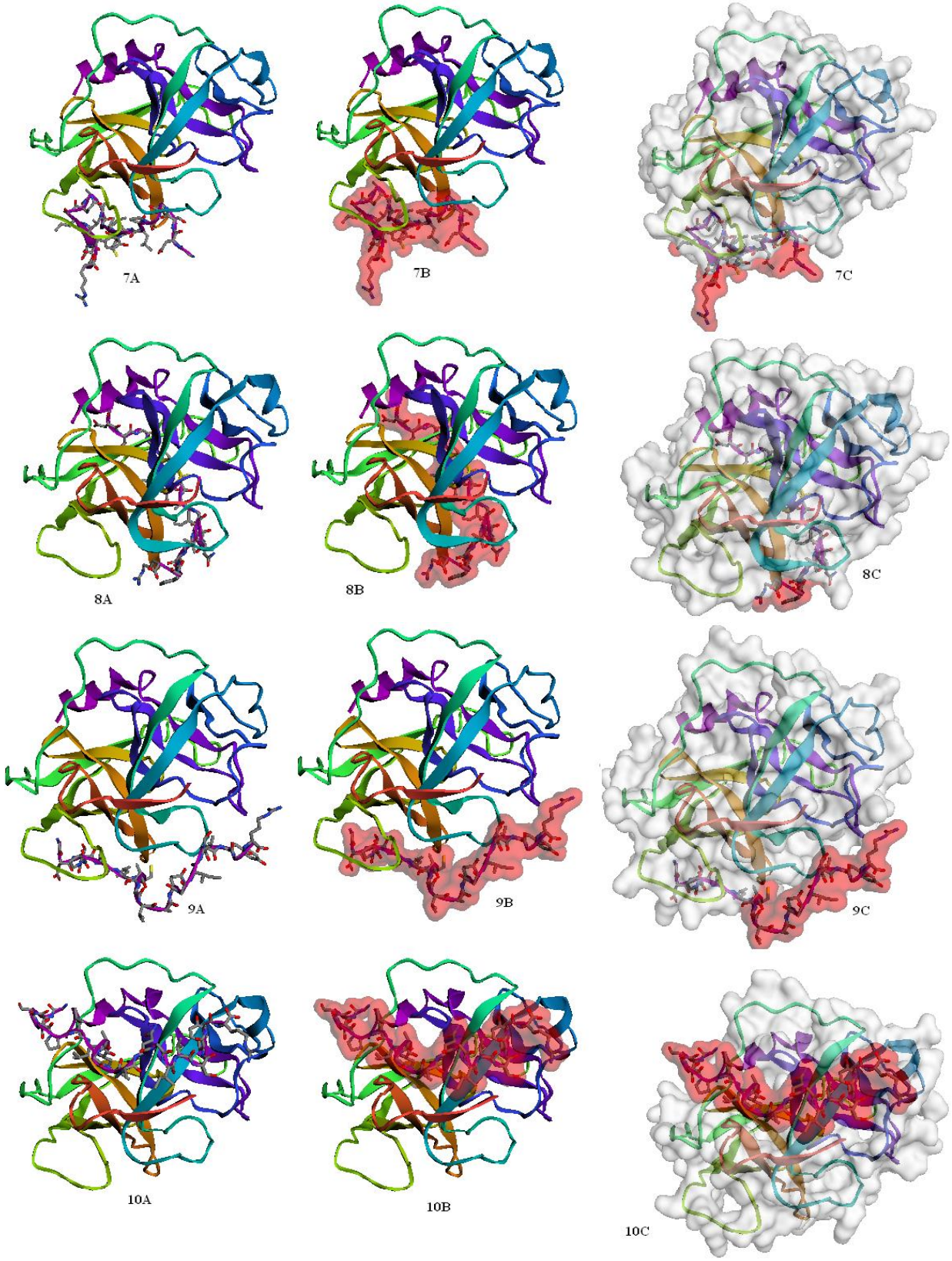

Figure: 2 Docking poses of top 10 best docked protein-peptide interactions with different poses (1-10 A-C)

3.2. Insilico peptidomimetics studies of the Novel peptide

Identification of novel mutations present in chymase enzyme that show disease progression in

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asthma and other allergic diseases in the human. There is mutagenesis observed in Arg 143 and Lys 192 in chymase that does not prefer the acidic P2 residue in asthma. The other sequence conflicts such as 28cys-Ser and 131-132 FP- AV AA and natural variants 46Gly-Arg, 66His-Arg and 98 Arg-His has direct mutagenesis in asthma. A peptidomimetic is an advanced methodology that mimics the natural protein and is used for pharmacophore analysis. The REFERENCES peptide EVKLRLMDPQACSHFRDFDHNLQLCVGNPRKTKSAFKGDSGGPLLCA GVA and novel peptide GDSGGPLLCAGVAQGIVSYGR sequences along with 3D structures had done protein-peptide docking using HDOCK server. Further have screened the best mimetic property of novel peptide and chymase protein that can predict using the SuperMimic software. The secondary structure of mimetic peptides was designed by inserting the suitable spacers at non-hotspot residue positions 18-20, 8-10 and another novel peptide by replacing the respective mutagenesis. There are two types of peptidomimetic drugs have been used to screen such as single spacers and multiple spacers. In addition, the single spacer subset of peptidomimetic has considered 21 residues and 50 residues (Table: 7). Further, they also showed interactions with C-terminal residues of Chymase mainly 146-211 amino acid residue thereby probably locking chymase function. Thus in our analysis, in addition to novel peptides, these set of peptidomimetics were found to replicate similar chymase substrate inhibitory interactions within asthma disease (Supplementary Table).

Table: 7. Peptidomimetic of type-1 spacers on chymase binding

No.	protein	stem_N	stem_C	Mimetic	Conformation	RMSD
1	Novel	18	20	AH-3	8	0.121
2	Novel	8	10	AH-1	7	0.129
3	Novel	5	8	AH-11	9	0.130
4	Novel	11	13	AH-1	4	0.135
5	Novel	12	14	AH-1	4	0.152
6	Novel	13	15	AH-1	4	0.182
7	Novel	32	33	AH-5	1	0.187
8	Novel	12	13	AH-5	1	0.187
9	Novel	44	46	AH-1	4	0.188
10	Novel	4	6	AH-3	2	0.192
11	Novel	9	11	AH-1	4	0.197
12	Novel	44	45	AH-5	1	0.198
13	Novel	43	45	AH-1	4	0.199
14	Novel	9	10	AH-5	1	0.200
15	Novel	13	14	AH-5	1	0.201
16	Novel	11	12	AH-5	1	0.222
17	Novel	10	12	AH-1	2	0.236
18	Novel	46	47	AH-5	1	0.242
19	Novel	10	11	AH-5	1	0.248
20	Novel	12	15	AH-6	10	0.259
21	Novel	45	46	AH-5	1	0.261
22	Novel	14	15	AH-5	1	0.261
23	Novel	19	21	AH-1	6	0.270
24	Novel	40	42	AH-6	1	0.270
25	Novel	32	35	AH-11	3	0.324

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26	Novel	28	31	AH-1	1	0.350
27	Novel	24	26	AH-3	6	0.372
28	Novel	38	42	AH-11	1	0.386
29	Novel	25	27	AH-3	6	0.396
30	Novel	41	45	AH-3	8	0.516

4. CONCLUSION

In the current research is targeting the function of chymase enzyme in asthma and design a novel peptide that inhibits the functional chymase activity within vasoactive peptide generation, extracellular matrix degradation in lung tissue. We have identified the structural property of chymase protein along with physic-chemical properties and secondary structures to build the 3D structure. We have designed the novel target peptides and predicted the mass that functionally selected the mutational sites of the target protein. We predicted 95 clusters of novel peptides that significantly have chymase protein interactions. Finally, have selected top-ranked 10 peptides was docked with the target protein. Our Insilico analysis reveals relatively stable binding and inhibitory interactions of top-ranked 10 peptides with chymase protein. We predicted peptidomimetic inhibitor of top-ranked peptides interacted that might serve as a set of novel chymase inhibitor in asthma and other chymase related allergic diseases. Our work thus provides great scope for experimental validations. These experimental studies would certainly help validate the novel therapeutic function of novel peptides as an asthma treatment and aid its clinical relevance.

CONFLICT OF INTEREST

All authors have declared that they have no conflict of interest.

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COMPUTATIONAL APPROACHES AND MOLECULAR DOCKING OF TARGET NATURAL CHEMICALS FOR TARGET AS GLUTATHIONE-S-TRANSFERASE

Swetha Sukla[1], Padmaja[1], Arghni[1], Prashantha C N[*1], Guruprasad NM[1], R.Ramachandra[1]
[1]Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560092, Karnataka, India

ABSTRACT: The aim of this study was to investigate the functions of glutathione-s-transferase (GST) in different tissues, particularly in tissue malignancies, inflammatory, myocardial infarction and stroke. In order to regulate tissue specific gene expression in different diseases shows increased oxidative stress and damages nerve cells. A series of mutations in GSTP1, k-ras and p53 genes inhibit MAPK pathways that regulate cell proliferation and cell death. The mutational analysis of GST has interlinked aberrant expressions with different diseases which show catalytic activity in biochemical pathways. Multiple functions of GST have been studied in different signaling and catalytic pathways and efforts are being made to develop potential drug targets with novel small molecular inhibitors. We used different natural inhibitors with polymorphic GST using molecular docking. Our findings suggest that natural inhibitors strongly bind with GST active sites and control different disease conditions.

KEYWORDS: Glutathione, cytosine, Amino acids, Oxidative stress, Myocardial Infarction, stroke, inflammatory diseases, cancer, Gene mutations

Corresponding Author: Dr.C.N.Prashantha
Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560092, Karnataka, India
E-Mail: prashanthacn@gmail.com;

1. INTRODUCTION

Glutathione-s-transferases (GST, EC 2.5.1.18) are a group of phase-II enzymes, associated with xenobiotic detoxification and involved in intracellular signaling pathways [1]. GST is distinctly classified into three major families namely the mitochondrial GST, microsomal GST or MAPEG (membrane associated proteins involved in eicosanoid and glutathione metabolism) and cytosolic GST [2,3]. Structurally, the cytosolic and mitochondrial GST show certain similarities [4,5] but are distinct from the MAPEG GST [3]. Based on sequence homology, kinetic properties, subunit structure and immunological cross-reactivity, the cytosolic GST is further classified into seven classes: (μ) mu, (α) alpha, (Ω) omega, (ζ) zeta, (π) pi, (θ) theta and (Σ) sigma [6-8]. There is growing interest in GST as potential therapeutic targets as they are predominant in various tumors. Furthermore, the overexpression of GST in cancerous tissues has been associated with multidrug resistance [9]. GST has been reported to play a vital role in various biosynthetic pathways such as biosynthesis of prostaglandins [10], steroids [11] and leuckotrines [12]. Recent findings suggest that GST is also involved in cell signaling pathways that include modulating cell proliferation and Apoptosis [13, 14, 9].

Apart from role of GST as enzymes where GST conjugates with GSH, they also play non-enzymatic roles by binding to a wide variety of nonsubstrate molecules like heme, bilirubin and a number of steroids. The role of GSTs in controlling cell proliferation and Apoptotic cell death has been studied in much detail in the recent times. Furthermore, there is evidence to show association of GST with mitogen-activated protein kinase (MAPK) pathways, where GST is involved in sequestering the kinase in complex early on, inhibiting its activity on

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other targets in the pathway. In line with this, JNK, a type of MAP kinase plays a role in a number of cellular functions such as apoptosis, inflammation, stress response and cell differentiation and proliferation. It has been reported that GST-pi interacts directly by way of protein-protein interaction with c-Jun-N-terminal kinase (JNK) and inhibits its activity. Some of the ways the JNK protein can be activated are stress stimuli, Ultra-violet (UV) radiations and protein synthesis inhibitors. Once activated, the JNK phosphorylates c-JUN, an activator protein-1 transcription factor (AP-1), resulting in the induction of AP-1-dependent genes which in turn play a role in cell proliferation and Apoptosis.

Prostaglandins with a cyclopentonone ring constitute substrates of GST in GSH conjugation reactions. Notably, GST also metabolize endogenous lipid mediators, which may have varied biological consequences. GST act as regulators in these pathways, as they affect the synthesis and elimination of eicosanoids. 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ_2), a metabolite of prostaglandin PGD_2, which plays a significant role in transcription factor activation, peroxisome proliferator-activated receptor γ (PPARγ). Furthermore, adipocyte differentiation is also regulated by PPARγ. The overexpression of GST interferes with efficient gene expression, by inhibiting the binding of activating ligand, 15-d-PGJ_2 and transcription factor, PPARγ. 15-d-PGJ_2 also plays a vital role in induction of gene expression through the antioxidant response element (ARE) by stimulating nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) [16]. 15-d-PGJ_2 has the ability to modify cysteine residues on Kelch-like ECH-associated protein 1 (Keap1), thus preventing proteosomal degradation of Nrf2 by Keap1. Here, GST stimulates the ARE-gene factory and indirectly regulates Nrf2 expression [17]. Consequently, GST is involved in controlling the antioxidant levels and other drug metabolizing enzymes in the Nrf2/Keap1 pathway. Further reports reveal that GST are involved in regulation of Phase-I, Phase-II, Phase-III detoxification enzymes, chaperones, ubiquitin-proteosome components, inflammation-associated proteins and apoptosis associated-proteins [9].

A high degree of genetic variations have been reported in GST [18-20]. Various reports have implicated that polymorphisms influence susceptibility to cancers [21, 22]. It may be interesting to explore how polymorphism of GST, play roles in altering the cancer pathways that affects different proteins and how individuals respond to drugs. The rationale of the current study is based on understanding the underlying mechanisms that may be involved in GST signaling pathways, which eventually result in pathogenesis of a host of diseases such as cancer, lung diseases, stroke, inflammatory diseases and myocardial infractions. In this paper, a computer based approach has been used to study the signaling pathways of GST.

2. MATERIALS AND METHODS

Initial characterization of phosphorylated and unphosphorylated x-ray crystallographic data gave information on GST protein three dimensional structure. The x-ray data of GST were collected from PDB database (PDB ID: 1K3O) [23]. The cytosolic GST is involved in xenobiotic metabolism, as well as in modulation of oxidative stress. The structure has 1.3 A^o of resolution predicts the complexicity of protein structure and stereochemical activity of peptide bonds were geometrically analyzed by using PROCHECK.

2.1. Active site prediction

Based on resolution of protein structure, predicts the possible ligand binding sites were searched using CastP calculation [24]. Some time the whole structure is also used for ligand binding property. The active site amino acids bind to most favorable hydrophobic probes with favorable binding energy and the resultant protein structure is used for molecular docking.

2.2. Ligand preparation

The structures of natural ligands such as Lycopene, glutathione, ellagic acid, ethacrynic acid,

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quercetin, caffeic acid, ferulic acid, porphyrin, curcumin, cinnamic acid, and also α-tocopherol bound ligand molecules were selected from PubChem compound database (http://pubchem.ncbi.nlm.nih.gov/search/search.cgi)[25]. Using pharmacokinetic properties include structural screening, fragment-analysis, and SAR facilitated to probe the parent library. The fragments were identified on the basis of "Lipinski's Rule of Five" that represented a suitable strive for proficient lead compounds. The training sets of lead molecules were generated through conformational search module and additional implementation has been done by Hyperchem skilled seven.0. For each compound, the systematic conformational search was attained by energy minimization by starting with 1000 to 2000 initial geometries at random torsion angle values. These were exclusively described exocyclic single bonds and chemical bonds within nonaromatic cycles. These ligand molecules can be used as potential drug molecules.

2.3. Molecular Docking

The docking analysis of existed ligands with GST proteins was carried out by means of the AutoDockTools (ADT) v 1.5.4 and AutoDock v 4.2 programs; (Autodock, Autogrid, Autotors, and Copyright-1991e2000) from the Scripps Research Institute, http://www.scripps.edu/mb/olson/ doc/autodock. To run AutoDock to search autogrid by creating Grid maps of different grid points and keeping ligands to cover binding pockets based on active site amino acids within the selected protein. A set of Lamarckian genetic algorithm was substantially used for molecular docking simulations. The simulation is carried about 10 times which ultimately yielded 10 docked conformations. From this, the lowest energy conformations were regarded as the best binding conformations. In the end, the reverse validation processes ensured the identified hits that fitted with generated pharmacophore models and active sites of both targets. Since all the parameters were required for molecular docking and Pharmacophore mapping were consequently fixed and used in regular process.

3. RESULTS AND DISCUSSION

Glutathione s-transferase is associated with xenobiotic binding and catalytic activity in metabolism, as well as in modulation of oxidative stress. The protein structure has two chains such as GST A1 N terminal domain (3-83 amino acids) and GST A1 C terminal (85-207 amino acids), there are two glutathione binding sites present in Val54 and Pro55 and Glu67 and Thr68 position of amino acid chain. The polymorphism of protein sequences shows Thr19 to Iso19, Pro113 to Glu113 and Lys117 to Glu117 amino acids and is potentially shows different disease conditions. The mutagenesis is present in the amino acid 71 Ile to Ala shows significant association of enzymatic activity and reduces protein stability. Another mutagenesis is present in 216Ala to His that hydrolyze S-glutathionyl benzoate to glutathione and benzoic acid. The resultant protein structure is used for homology modeling and drug docking studies against different diseases.

Molecular modeling and simulation techniques were employed to analyze the protein structure superimposition and were predicted using SwissPDBViewer. The geometric accuracy of GST structure was predicted using SAVS and the results are predicted using ramachandran plot. The structural stereochemical activity of peptide bonds shows 99.50% of verify 3D and 91.361 % of quality factor that shows the selected GST structure shows best complex with three dimensional structures. We used active sites prediction of selected protein using Q-site finder. There are 28 active site amino acids and has 18 binding pockets. The active site amino acids are listed in table 1.1 and the structure is predicted in **figure 1**.

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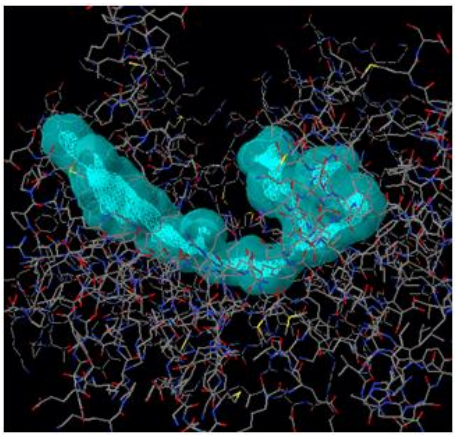

Fig 1: Active Site amino acids represented using CastP Calculation

Table 1: Active site amino acids were predicted using CastP

Amino Acids	Coordination
Arg15, Ser18, Met51, Phe52, Val54, Pro55, Val66, Glu67, Thr68, Arg69, Ala70, Leu72, Asn73, Tyr82, Asp93, Ile96, Glu97, Ile99, Ala100, Leu102, Glu104, Leu107, Arg155, His159, Leu160, Glu162, Leu163 and Tyr166	Min Coords: (58, 8, -1) Max Coords: (93, 38, 36)

In the present study, natural products such as Glutathione, Caffeic acid, Ferulic acid, Cinnamic acid, Ethacrynic acid, Curcumin, Cibracon blue, Quercetin and Tocopherol are retrieved from Pubchem compound database and the resultant compounds were used for molecular docking. Docking simulations of active site protein 1K3O were performed using AutoDock and AutoGrid which has performed successfully to reproduce experimentally small interaction energy shows lowest docking energy. The docking was performed on target protein with natural products and the results are interpreted using Molegro molecular Viewer.

Table 2: Molecular docking studies of GST Enzyme (1K3O) with natural Ligands

Sl. No.	Ligand	H.Bonds	Inhibitory Constant (µM)	Electrostatic Energy (Kcal/Mol)	RMSD	Binding Energy	Amino Acids
1.	Glutathione	3	796.03	-2.45	72.58	-4.23	LYS127, ARG131
2.	Caffeic acid	3	60.28	-0.89	85.27	-5.76	LYS127, ARG45, VAL55
3.	Ferulic acid	3	161.03	-2.20	90.44	-5.17	ASN80, TYR82, ARG89

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4.	Quercetin	2	7.06	-0.20	72.59	-7.03	UNO*
5.	Ethacrynic acid	2	15.62	-0.67	85.16	-6.56	VAL55, LYS127
6.	Curcumin	1	29.94	-0.21	66.67	-6.17	ARG13
7.	Cibracon blue	6	6.62	-3.72	85.71	-7.07	ARG15, GLN54, ARG69, LYS127, ARG131
8.	Cinnamic Acid	2	137.66	-0.91	73.75	-5.27	THR68, ARG15
9.	Tocopherol	1	1.21	0.04	78.58	-3.98	ARG89

*Internal energy present within the ligand molecule

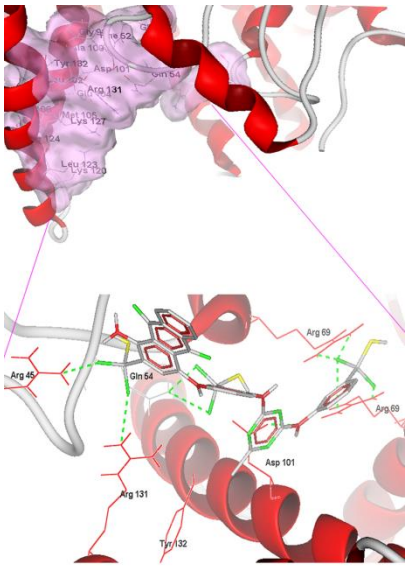

Figure 2: Active Site Depiction: Binding of Cibracon blue at the active site of GST (1K3O)

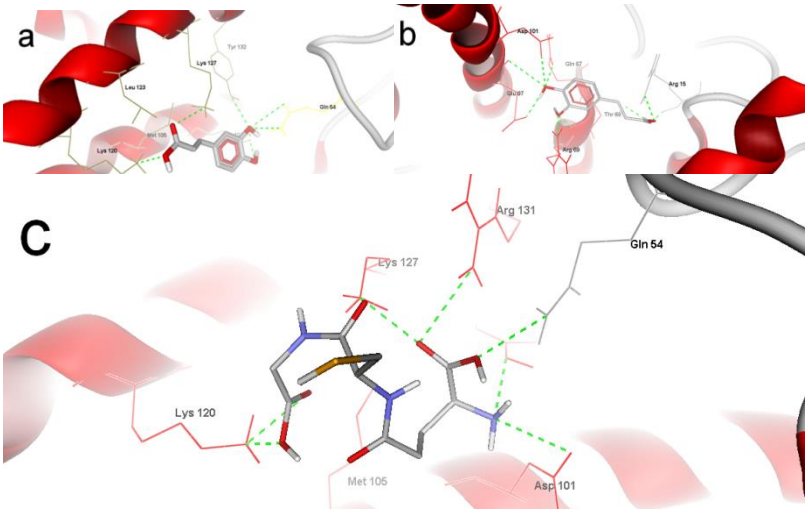

Figure 3: Docked poses of ligands with GST (1K3O) depicting the active site residues along with the H-bond interactions (dotted green line); Ligands: (a) Caffeic acid; (b)Ferulic acid; (c) Glutathione.

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The **figure 3** shows the results of molecular docking on GST with natural compounds. The interaction of cibracon blue with GST protein shows strong interaction by forming 6 hydrogen bonds with interaction energy of -7.07 kcal/mol. The atoms present in between the interaction energy shows C2:H1 atoms binds to active site amino acids ARG15:H1, GLN 54:H1, ARG69:H1, LYS127:H1, ARG131:H1 molecules. These amino acids are potentially targeted as active sites of cibracon blue interaction. The other compounds such as glutathione, caffeic acid and ferulic acid show 3 hydrogen bond interactions with GST with interaction energy of -4.23, -5.76 and -5.17 kcal/mol respectively, forming good interaction energy with active site amino acids LYS127, ARG131, ARG45, VAL55, ASN80, TYR82 and ARG89. The active site amino acid such as LYS127 and ARG131 shows glutathione binding sites in active site protein position and is potentially targeted as the best intermolecular atoms between the protein and ligand. Other natural compounds such Quercetin, Ethacrynic acid, Curcumin, Cinnamic Acid and Tocopherol show weak interactions with the target protein and are not potentially used for inhibition of GST in different biological mechanism.

4. CONCLUSION

The present study demonstrates the interaction of GST with natural inhibitors that can show a down regulation in different signaling pathways to inhibit different diseases. The three dimensional structure of GST has two active sites in both N and C-terminal chains, having glutathione binding positions shows specific polar interactions between protein and ligand. Among the ligands used in this study, the natural inhibitor cibracon blue strongly bound with GST. This implies that it may regulate the downstream process of signaling pathways that can aide to control disease mechanisms. The molecular docking of simulated structural comparisons of binding energies on cibracon blue and other natural products shows RMSD of 85.71 and the electrostatic energies of non-bonded atoms in GST show -3.71 kcal/Mol which demonstrated the best interaction among the regulatory sites and may control the process of further signaling pathways. The overall results conclude that the interaction of natural compounds helps to regulate different types of diseased proteins and can inhibit the disease progression.

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THE IDENTIFICATION AND VALIDATION OF DIFFERENTIALLY EXPRESSED GENES OF BOTH MOUTH BUCCAL CAVITY AND NASAL CAVITY EPITHELIAL TISSUES OF LUNG CANCER

Akash Dnyanoba Adude[1], Rahul Dnyanoda Chandawar[1], Gajanan Balaji Gaikwad[1], Prashantha Nagaraja[1*], Kiran Raj T[2]

[1]Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560064

[2]Department of Biotechnology, PES Institute of Technology, Bangalore-560085

ABSTRACT: Tobacco smoking is most common in all over the world and many people is affecting lung cancer, in small amount of people is affecting with mouth buccal cavity and nasal cavity cancer. There is a morphological change in the respiratory tract that will affect airway epithelial cells they can undergo genetic alterations in lungs may cause lung cancer. We used microarray array analysis to predict and classify the genetic mutations and list of genes present in both buccal and nasal cavity epithelial cells. The differential expression of mouth buccal cavity shows 82 upregulated and 3747 down regulated genes, these genes is further classified by functional enrichment shows 35 transcriptional regulated genes in mouth epithelial tissues. The nasal cavity shows 1243 upregulated and 2979 down regulated genes, further functional enrichment shows 249 genes are in transcriptional regulation. We investigated the genes in mouth and nasal cavity by using gene ontology, and functional enrichment methods, and pathway prediction. All these results may facilitate clinicians and molecular diagnostic professionals to understand the genes in both never and current smoker genes in mouth buccal cavity and nasal cavity and are more invasive to diagnostic procedures for the patients.

KEYWORDS: Lung cancer, buccal cancer, oral cancer, microarray, R software

***Corresponding Author: Dr. C. N. Prashantha**

Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560092, Karnataka, India

E-Mail: prashanthacn@gmail.com;

1. INTRODUCTION

Lung cancer is a world most leading cancer type and is highest death rate in the world because of carcinogens of chemicals, tobacco smoking, and other genetic factors [1]. The rate of mortality on lung cancer is 17% in America [2]. About 1.5 million people are affecting every year and the lung cancer rate is very high in India about 1.85 million people are affected in India. Over 15% of people are diagnosed in early stage of infection. The death rate of lung cancer is very high compared to colon, breast and pancreatic cancer [3]. Currently there is no treatment for lung cancer in the initial stage and it can treat by surgery, chemotherapy, radiotherapy, and targeted molecular therapy can treat the disease but only one third of patients alone are cured the disease in early stage of infection. There is a poor prognosis to treat the disease only possible to cure the disease in early stages. However, there is a need to develop new treatments for lung cancer [4].

Tobacco smoke contain many chemicals and it will harm nearly every organ in the body it cause cancers of the larynx, mouth, throat and other organs [5]. Smoking that affects epithelial cells in mouth buccal cavity and nasal cavity that also transfers to respiratory tract. Recently, scientists have identified that tobacco smoke contain various toxic chemicals whose

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metabolites bind to DNA and induce activating point mutation in the p53 tumor suppressor and K-RAS gene [6]. In comprehensive study of genetics, the gene mutations in the p53 tumor suppressor and K-RAS proto-oncogene are frequent alterations in many human tumors [7,8]. In fact, oncogenic mutations of the K-RAS gene alone can cause cancer and this mutation is found in 30% of human lung adenocarcinomas [9, 10]. There are large gene expression data sets can provide novel insights into lung cancer [11, 12]. There are specific differences between lung cancer in smokers and nonsmokers, and none have compared nonmalignant to tumor tissue in the same subject [13].

The microarray data analysis helps to understand differential expression of diseases, developmental stages, cellular responses and biological phenomenon of smokers and non-smokers in mouth and nasal cavity. The gene expression signatures consisting of tens to hundreds of genes have been associated with many important aspects of the systems in mouth and nasal cavity [14]. The increased number of clinical trials demonstrates the targeted treatments that specifically benefit patients defined by molecular subtypes such as BMK1/ERK1 is observed in both smokers and non smoker lung cancer patients [15], as well as observed prognostic gene expression signature profiles or signatures to a broad range of biological categories. Although some of these methods include signaling pathways in their categories, their focus has not been on regulatory mechanisms that control the observed gene expression changes. In this study, we subsequently analyzing relationship between the gene expression in mouth buccal cavity and nasal cavity of both smoker and non-smoker, we evaluated the relationship between the gene mutations, gene expression changes and to predict gene expression biomarkers is ultimately predicted epithelial tissues.

2. MATERIALS AND METHODS

In order to study the relationship of smoker and non-smoker differences to get the non-small cell lung cancer and also to find the significant gene signatures and their associated gene signaling pathways from both mouth buccal cavity and nasal cavity epithelial gene expression datasets were predicted using microarray. There are two smoke exposing epithelial tissues such as mouth and nose. The extra thoracic epithelium of nasal and buccal cavity healthy current and never smoker mRNA samples is hybridized using Affymetrix method (GSE8987) [16]. The raw dataset contains 25 samples of which 10 samples are mouth buccal cavity epithelium tissues mRNA samples and 15 Nasal epithelial samples from never smoker and current smoker. Using annotation platform of these two samples with GPL96 (hgu133a) of mouth buccal cavity mRNA samples having 22283 probes and GPL571 (hgu133a2) of nasal cavity mRNA samples of 22277 probes were used for prediction of differential expression of genes.

2.1 Preprocessing and Normalization

The raw data of selected gene expression dataset is carried by Affymetrix method and the data analysis is carried by R and BioConductor packages. There are several gene expression analysis packages contains biological algorithms that can help to predict differential gene expressions. While comparing the two different sample sets is hybridized to the same array at systematic adjustments of intensity effects. To correct intensity values of background correct, log2 transformation and quantile normalization using RMA algorithm and summarization of corrected data with standard methods is implemented by MAS 5 (Affymetrix Microarray Suite) method. The GCRMA method is used to filter the log2 intensity values based on intensity of mean, median and standard deviation calculations.

2.2 Differential gene expression analysis

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The significant quality filtered probe sets of all datasets is used to predict differential expression analysis by LIMMA package. Limma is especially designed to predict linear models of datasets assigned with differential expressions. To extract the datasets based on sample sets and conditions of samples are aligned and calculate differential expressions by empirical Bayes shrinkage of the standard errors towards a common value by computing the moderated t-statistics, moderated F-statics and log-odds. To create topTable of n number of differentially expressed probes for any contrasts is imposed by fold change cutoff and can see how many genes are returned by using ifc modifier for topTable. Then to create the list of genes with adj.P.Val ≤ 0.05 and fold change ≥ 2 for the first contrasts that can make heatmap of the expressions. In order to annotate the probesets into gene symbols need to load associated databases package and annotate package that can extract the probesets ID's from the topTable results, and match the symbols.

2.3 Functional Annotation and Enrichment analysis

The differential expressed genes are annotated with REFERENCES genome database to predict the gene names based on Affymetrix probe ids. Using DAVID functional annotation database to predict the gene names and functions based on clusters and to convert the gene names based on annotation platform. The Gene ontology database also used to predict the functions of each gene and their associated networks that can predicted in large scale genomics and FDR = 0.05 is used as cut-off criterion. The functional enrichment analysis is predicted based on GO functional terms is compared with process, function and component of different co-expressions that can be predicted by using Gorilla functional enrichment analysis tool, ToppFun and FunRich Tools.

2.4 Protein-protein interaction network analysis

Using String database is used to predict the functionally enriched genes and their associated protein functions is predicted through online. The information of human PPI network relationship genes and associated proteins related to the disease is collected to construct PPI network to classify most novel genes that significantly associated the lung cancer. The differential expression of genes were mapped to the String database and then known and predicted associations were scored and integrated. The combinations of genes with threshold are scored >0.4 of median confidence to visualize maximum score and the results is observed in Cytoscape software.

3. RESULTS AND DISCUSSION

The raw data of mouth buccal cavity and nasal cavity epithelial tissue samples were used for preprocessing and normalization using Affy package. The signal extraction methods such as MAS5, RMA and GCRMA methods to preprocess, normalization and quality analysis of different intensity values that is screened by log2 intensity calculation. The differential expression of mouth epithelial dataset has 22283 probesets is filtered by using normalization shows 13411 probes is accepted with logP intensity ranges. The redundant log2 intensity values are final filtered shows 8804 genes were predicted in differential expressions.

The nasal datasets has $numLowvar has 11108 is screened based on log2 transformation is filtered by 11107 genes is used as differential expressions. We have designed the matrix of each individual samples by contrast matrix to design coefficients (Never smoker-Current smoker). We selected the sample sets of condition column represent only 4 coefficients and the coordinated of each topTable are calculated by empherical bayes method. Here, we predicted the 82 upregulated and 3747 down regulated genes is predicted in mouth epithelial tissues. Further we have predicted the functional enriched genes of both tissues shows only

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35 transcriptional genes is expressed in transcriptional regulation of which only 14 genes is most commonly involved in different signaling pathways in mouth epithelial tissues. The nasal epithelial tissues has 1243 upregulated and 2979 down regulated genes of which 345 genes is involved in different functional mechanism, biological process, and cellular component, out of these genes only 249 genes is involved in transcriptional regulation in different signaling pathways shows only 69 genes is involved in lung cancer associated signaling pathways. Further study has done to predict the potential drug targets of these two epithelial tissues shows only 14 genes in mouth epithelial tissues and 54 genes in nasal epithelial tissues are used as potential drug targets.

Table: 1a Contrast matrix and coefficient of mouth buccal cavity epithelial samples to predict differential expressions

Coefficients (Coef)	Fold Change (IFC)	No of genes	Up	Down
Coef=1 (Mouth1- Mouth2) Never smoker- Current smoker	1	853	1	852
Coef=2 (Mouth1 – Mouth3) Never smoker – Current smoker	1	1265	26	1239
Coef= 3(Mouth1 – Mouth4) Never Smoker – Never Smoker	1	1597	65	1532
Coef=4 (Mouth2 – Mouth3) Current Smoker – Current Smoker	1	6	0	6
Coef=5 (Mouth2 – Mouth4) Current Smoker – Never Smoker	1	76	14	62
Coef=6 (Mouth3 – Mouth4) Current Smoker – Never Smoker	1	72	16	56

Note: Coefficients (Coef) represents the selection of matrix based on type of sample comparison, Fold Change (IFC) the fold change represents the probability of samples selection based on differential expression,

Table: 1b Contrast matrix and coefficient of Nasal epithelial samples to predict differential expressions

Coefficients (Coef)	Fold Change (IFC)	No of genes	Up	Down
Coef=1 (Nose1- Nose2) Never smoker – Current Smoker	1	1	0	1
Coef=2 (Nose1 – Nose3) Never Smoker – Never Smoker	1	2255	414	949
Coef= 3(Nose1 – Nose4) Never Smoker – Current smoker	1	1608	193	162
Coef=4 (Nose2 – Nose3) Current Smoker – Never Smoker	1	2783	398	997
Coef=5 (Nose2 – Nose4) Current Smoker – Current Smoker	1	2259	238	205
Coef=6 (Nose3 – Nose4) Never Smoker – Current Smoker	1	665	0	665

Note: Coefficients (Coef) represents the selection of matrix based on type of sample comparison, Fold Change (IFC) the fold change represents the probability of samples selection based on differential expression,

In regulatory study shows there are 10 genes is most commonly present in lung adenocarcinoma such as ***CDKN1B, MTUS1, MID1, EMP1, VDR, IGF2BP3, NEDD4L, HPGD, TMPRSS2 and ZFAND5*** genes is highly expressed in mouth epithelial current smoker and never smoker datasets. Further we have predicted the pathways shows there are

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14 genes is involved in different signaling pathways such as ***S1P1 pathway, GMCSF-mediated signaling events, IGF1 pathway, EGFR-dependent Endothelin signaling events, Signaling events mediated by Hepatocyte Growth Factor Receptor (c-Met), CDC42 signaling events, Plasma membrane estrogen receptor signaling, Thrombin/protease-activated receptor (PAR) pathway, Syndecan-1-mediated signaling events*** and ***Glypican 1 network*** these genes are ***VDR, ASAP2, FOXO4, KAT2B, CTNNA1, TMPRSS2, CDKN1B, MDF1C, NEDD4L, ACTR2, MYO6, ZFAND5, ABI1 and EZR*** genes is most common in non-small cell adenocarcinoma in both smoker and never smoker samples.

Table: 2a. Functional enrichment analysis of topTable upregulated genes on never smoker – current smoker samples comparison of Mouth buccal cavity epithelial tissues.

Term	%	P-Value	Genes	Fold Enrichment	FDR
negative regulation of cell proliferation	14.285	0.00126	VDR, KAT2B, CDKN1B, NUPR1, SKAP2, FOXO4	7.026315	1.7962
cellular response to insulin stimulus	7.1428	0.01053	KAT2B, FOXO4, LPIN1	18.65073	14.100
steroid metabolic process	9.5238	0.01084	OSBPL2, FDFT1 CYP1B1, CYP1A1,	8.371287	14.476
toxin metabolic process	4.7619	0.01140	CYP1B1, CYP1A1	169.1	15.176
response to insulin stimulus	7.1428	0.02188	KAT2B, FOXO4, LPIN1	12.682	27.201
response to organic substance	14.285	0.02276	CYP1B1, KAT2B, CYP1A1, DUOX2, FOXO4, LPIN1	3.51803	28.138
cell cycle arrest	7.1428	0.02312	CDKN1B, KAT2B, FOXO4	12.3131	28.515
hormone metabolic process	7.1428	0.02439	CYP1B1, CYP1A1, DUOX2	11.9646	29.837
regulation of cell proliferation	14.285	0.03175	VDR, CDKN1B, KAT2B, NUPR1, SKAP2, FOXO4	3.22299	37.059
cellular response to hormone stimulus	7.1428	0.03704	KAT2B, FOXO4, LPIN1	9.535714	41.818
peptidyl-lysine modification	4.7619	0.03827	KAT2B, DOHH	49.73529	42.872
isoprenoid biosynthetic process	4.7619	0.04487	CYP1A1, FDFT1	42.275	48.250
regulation of hormone levels	7.1428	0.04660	CYP1B1, CYP1A1, DUOX2	8.399006	49.574
response to peptide hormone stimulus	7.1428	0.04827	KAT2B, FOXO4, LPIN1	8.23538	50.829
hydrogen peroxide metabolic process	4.7619	0.05578	CYP1A1, DUOX2	33.82	56.115
oxidation reduction	11.904	0.05650	CYP1B1, CYP1A1, DOHH, DUOX2, FDFT1	3.307902	56.589
regulation of microtubule	4.7619	0.06872	CDKN1B, MID1	27.27419	63.994

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polymerization or depolymerization					
regulation of cell size	7.1428	0.08046	CDKN1B, NUPR1, CDKN2AIP	6.156553	69.984
regulation of microtubule cytoskeleton organization	4.7619	0.09199	CDKN1B, MID1	20.13095	74.955
isoprenoid metabolic process	4.7619	0.09616	CYP1A1, FDFT1	19.21590	76.555
heme binding	7.1428	0.03359	CYP1B1, CYP1A1, DUOX2	10.05914	33.315
iron ion binding	9.5238	0.03642	CYP1B1, CYP1A1, DOHH, DUOX2	5.269074	35.592
tetrapyrrole binding	7.1428	0.03775	CYP1B1, CYP1A1, DUOX2	9.435319	36.643
aromatase activity	4.7619	0.05806	CYP1B1, CYP1A1	32.4575	50.801
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen	4.7619	0.06928	CYP1B1, CYP1A1	27.04791	57.315
cell adhesion molecule binding	4.7619	0.06928	EZR, CTNNA1	27.0479	57.315
electron carrier activity	7.1428	0.09725	CYP1B1, CYP1A1, DUOX2	5.50749	70.273
electron carrier activity	7.1428	0.09725	CYP1B1, CYP1A1, DUOX2	5.50749	70.273
oxygen binding	4.7619	0.09783	CYP1B1, CYP1A1	18.8706	70.502

Table: 2b Functional enrichment analysis of topTable upregulated genes on never smoker – never smoker samples comparison of Nasal cavity epithelial tissues

Term	**%**	**PValue**	**Genes**	**Fold Enrichment**	**FDR**
protein catabolic process	8.4812	1.28E-06	RAD23B, CUL5, TPP1, SPG7, ACE2, UBA5, PCNP, UBE3A, WWP1, HLTF, PJA2, UBE2G1, EDEM3, FEM1B, CD2AP, ARIH1, PSMB3, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, ADAM9, TBL1XR1, UBE2J1, ERLIN2, SOCS5, AFG3L2, CLPX, UBE2N, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46,	2.2481	0.0022

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			CAND1, SIAH2, CUL4B, RNF111, UBE2E1		
macromolecule catabolic process	9.8619	1.37E-06	RAD23B, SPG7, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ISG20, ZFP36L1, ARIH1, CUL5, TPP1, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, ADAM9, AGA, ABCE1, TBL1XR1, GUSB, UBE2J1, GTF2H3, ERLIN2, PCNP, SOCS5, AFG3L2, HLTF, CLPX, UBE2N, PJA2, DNASE2, UBE2E3, HSP90B1, UBR5, FBXL5, ACE2, RNF138, USP46, CAND1, SIAH2, CUL4B, UBE2E1, RNF111	2.0818	0.0023
protein transport	9.6646	1.59E-06	SEC24B, CHMP5, AP1AR, CCDC91, SNX4, PEX3, PDIA4, NXT2, CHMP2B, CEP57, ZFYVE16, AAGAB, NECAP1, TLK1, VPS16, SNX24, RAB6A, RANBP2, TNPO2, SAR1B, RAB27B, SEC24D, SEC61A1, RAB2A, SCAMP1, SEC23A, MCM3AP, STX4, ATG9A, LIN7C, NUP85, STXBP3, CLPX, EPS15, COG4, HSP90B1, ERBB2IP, IPO7, RAB22A, IPO5, YWHAQ, SDCBP, JAK2, SRP72, GGA1, SNX13, KPNA1, SERP1, F2R	2.0911	0.0027
establishment of protein localization	9.6646	2.06E-06	SEC24B, CHMP5, AP1AR, CCDC91, SNX4, PEX3, PDIA4, NXT2, CHMP2B, CEP57, ZFYVE16, AAGAB, NECAP1, TLK1, VPS16, SNX24, RAB6A, RANBP2, TNPO2, SAR1B, RAB27B, SEC24D, SEC61A1, RAB2A, SCAMP1, SEC23A, MCM3AP, STX4, ATG9A, LIN7C, NUP85, STXBP3, CLPX, EPS15, COG4, HSP90B1, ERBB2IP, IPO7,	2.0720	0.0035

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			RAB22A, IPO5, YWHAQ, SDCBP, JAK2, SRP72, GGA1, SNX13, KPNA1, SERP1, F2R		
protein localization	10.059	1.79E-05	SEC24B, CHMP5, AP1AR, PEX3, PDIA4, ZFYVE16, AAGAB, NECAP1, TLK1, RANBP2, RAB6A, VPS16, SAR1B, RAB27B, SEC24D, SEC23A, SCAMP1, MCM3AP, STX4, ATG9A, G3BP2, STXBP3, NUP85, CLPX, IPO7, IPO5, SDCBP, SRP72, SNX13, KPNA1, SERP1, SNX4, RDX, CCDC91, CHMP2B, NXT2, CEP57, SNX24, TNPO2, SEC61A1, RAB2A, LIN7C, EPS15, COG4, HSP90B1, ERBB2IP, RAB22A, YWHAQ, JAK2, GGA1, F2R	1.8803	0.0310
proteolysis involved in cellular protein catabolic process	7.6923	1.83E-05	RAD23B, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ARIH1, CUL5, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, ADAM9, TBL1XR1, UBE2J1, ERLIN2, PCNP, SOCS5, HLTF, CLPX, UBE2N, PJA2, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46, CAND1, SIAH2, CUL4B, RNF111, UBE2E1	2.1137	0.0318
cellular protein catabolic process	7.692	2.05E-05	RAD23B, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ARIH1, CUL5, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, ADAM9, TBL1XR1, UBE2J1, ERLIN2, PCNP, SOCS5, HLTF, CLPX, UBE2N, PJA2, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46, CAND1, SIAH2, CUL4B, RNF111, UBE2E1	2.1032	0.0356

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cellular macromolecule catabolic process	8.6785	2.56E-05	RAD23B, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ISG20, ZFP36L1, ARIH1, CUL5, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, ADAM9, ABCE1, TBL1XR1, UBE2J1, GTF2H3, ERLIN2, PCNP, SOCS5, HLTF, CLPX, UBE2N, PJA2, DNASE2, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46, CAND1, SIAH2, CUL4B, RNF111, UBE2E1	1.9735	0.0443
modification-dependent macromolecule catabolic process	7.2978	3.76E-05	RAD23B, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ARIH1, CUL5, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, TBL1XR1, UBE2J1, ERLIN2, PCNP, SOCS5, HLTF, UBE2N, PJA2, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46, CAND1, SIAH2, CUL4B, RNF111, UBE2E1	2.0961	0.0652
modification-dependent protein catabolic process	7.2978	3.76E-05	RAD23B, UBE3A, UBE2G1, UBA5, EDEM3, FEM1B, CD2AP, ARIH1, CUL5, PSMB3, WWP1, FBXO28, PSMD2, RNF11, ZMPSTE24, FBXW12, RANBP2, FBXO3, TBL1XR1, UBE2J1, ERLIN2, PCNP, SOCS5, HLTF, UBE2N, PJA2, UBE2E3, HSP90B1, UBR5, FBXL5, RNF138, USP46, CAND1, SIAH2, CUL4B, RNF111, UBE2E1	2.0961	0.0652
regulation of cellular protein metabolic process	6.1143	1.47E-04	GCLC, IL6ST, FEM1B, TIMP1, ZFP36L1, PRKAR2B, APP, PRKAR2A, MDFIC, PSMB3, RB1CC1, ITGAV, PSMD2, PUM2, INSR, ADAM9, EIF2B5,	2.1267	0.2543

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			IBTK, PAIP1, SMAD4, NDFIP1, UBE2N, MAP4K5, EIF4E, EP300, HIPK3, PRKAR1A, JAK2, EIF2AK3, BMPR1A, UBE2E1		

The overall results of functional enrichment shows that there are 3241 genes are differentially expressed based on molecular and biological functions. Out of these genes only 345 genes top regulated genes in nasal epithelial tissues of both never smoker and current smoker tissues. Further to predict the transcriptional regulatory genes of all 345 genes shows only 249 genes is involved in transcriptional factors that can regulate the expression of different signaling pathways such as mTOR signaling pathway, TRAIL signaling pathway, Arf6 signaling events, Arf6 downstream pathway, PAR1-mediated thrombin signaling events, IFN-gamma pathway, PDGFR-beta signaling pathway, GMCSF-mediated signaling events, Internalization of ErbB1 and Nectin adhesion pathway shows 69 genes is differentially expressed and these genes is further used to predict drug sensitive to non-small cell lung cancer shows 54 genes is used as a potential drug targets for nasal epithelial tissues in lung cancer.

4. CONCLUSION

The overall analysis of mouth epithelial tissues and nasal epithelial tissue respiratory tract non-small cell lung cancer is predicted to identify the differential expressed genes based on the molecular mechanism, biological process and cellular component. Here, we predicted the 82 upregulated and 3747 down regulated genes is predicted in mouth epithelial tissues. Further we have predicted the functional enriched genes of both tissues shows only 35 transcriptional genes is expressed in transcriptional regulation of which only 14 genes is most commonly involved in different signaling pathways in mouth epithelial tissues. The nasal epithelial tissues has 1243 upregulated and 2979 down regulated genes of which 345 genes is involved in different functional mechanism, biological process, and cellular component, out of these genes only 249 genes is involved in transcriptional regulation in different signaling pathways shows only 69 genes is involved in lung cancer associated signaling pathways. Further study has done to predict the potential drug targets of these two epithelial tissues shows only 14 genes in mouth epithelial tissues and 54 genes in nasal epithelial tissues are used as potential drug targets. This study demonstrates that using computational empherical bayes method to discover both tumor and control tissues expressions in lung cancer patients. This research helps clinicians and molecular diagnostic professionals to understand the genes involved in both never smoker and current smoker genes in NSCLC and are more invasive to diagnostic procedures for the patients. We hope that our results will encourage researchers to make understand about tissue types to predict the genes and make sure this data can use for public use. As more data becomes available, our models can be further improved, and future discoveries could be made in other cancers.

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MICROARRAY DATA ANALYSIS TO PREDICT BRCA1 MUTATED GENES IN BREAST CANCER

Prashantha C N[1], Ajay Jakhar[1], Swetha Reddy[1], Sukanya VK[2]

1. Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560064
2. Departments of Biotechnology, RV College of Engineering, Bangalore

ABSTRACT: Women with BRCA1 and BRCA2 gene mutations are at increased risk of breast cancer in comparison to those who do not carry the mutation in familial and somatic condition. Therefore, it is of interest to identify differentially expressed gene patterns related to BRCA1 and BRCA2 gene mutations that significantly expressed in breast cancer. We have developed microarray meta-analysis methods to predict differentially expressed gene levels between hereditary BRCA1 mutations linked with sporadic breast cancer, using statistical methods. SVM classifier to identify gene ranked and their associated gene networks help to classify gene profiles based on their functional enrichment. We have predicted 2381 up-regulated and 2057 down-regulated genes that significantly associated with BRCA1 and BRCA2 related gene mutations (P-value <0.01). We also predicted SVM classifiers to construct gene networks has 810 genes that significantly associated with 4 different types of which 592 genes that has gene expressions at metastatic condition. We further screened the genes based on functional enrichment shows 30 genes are significantly associated with GO terms and many signaling pathways, these genes us mainly used for potential drug targets.

KEYWORDS: Microarray, Breast cancer, Drug targets, drug discovery, gene signatures, Meta-analysis, BRCA1, BRCA2,

Corresponding Author: Dr.C.N.Prashantha
Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560092, Karnataka, India
E-Mail: prashanthacn@gmail.com

1. INTRODUCTION

Cancer is a genetically modified uncontrolled cell proliferation that has multistep process arising from gene mutations involved in signaling, cell-cycle and/or cell-death pathways resulting in a mis-regulation of the pathways [1]. These mutations produce an oncogene with a dominant function, and/or tumor suppressor genes causing a loss of function. Either way, the cell cycle is disrupted leading to uncontrolled cell division and growth – a common feature of every cancer type [2]. Breast cancer is a complex collection of diseases with characteristic clinical, histopathological, and molecular features [3].Breast carcinogenesis involves genetic and epigenetic alterations that cause aberrant gene function [4].The commencement and development of breast cancer are caused due to the gathering of genetic mutations which lead to abnormal cell functions [5]. These genetic mutations can be inherited or irregular and may lead to the activation of oncogenes and block tumor suppressor genes [6]. It is also shown that breast cancer can be caused by epigenetic alterations, which do not affect the primary DNA sequence but cause abnormal transcriptional regulation that causes a change in gene expression patterns involved in cellular proliferation, survival, and differentiation [7].Breast cancer is the most commonly occurring disease with around 1.38 million new breast cancer cases being diagnosed every year, globally [8]. It is also the most common cancer affecting women and stands second in causing cancer deaths among women [9] and fifth in cancer overall [10].The survival of breast cancer patients is mainly associated

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with two factors – early detection of disease and adjuvant systemic treatment which includes chemotherapy and hormone therapy [11]. Treatments are largely dependent on clinicopathological conditions that include age, tumor size, histological grade, estrogen receptor (ER), progesterone receptor (PR), and HER2 status [12]. Although all patients with breast cancer are treated with chemotherapy, only a few patients will benefit from it [13]. Selecting an appropriate adjuvant therapy for breast cancer patients depends on reliable predictive markers [14].

The microarray-based gene expression profiling has had a tremendous development in the past decade [15]. The discovery of this technology has been considered as a new dawn in cancer biology and oncology practice [16]. It has aided in the analysis of the multiple gene expression levels in a tumor sample [17].With many gene expression profiling studies performed in the last decade, the datasets are available for analysis and meta-analysis [18]. Microarray-based gene expression profiling studies have helped in understanding the complexity of breast cancer which is not a simple disease but a collection of many genetic mutations [19]. In this study, we used microarray-based gene expression profiling datasets of breast cancer from Gene Expression Omnibus (GEO) database to come up with significant gene set that can be used as an initial set in developing predictive and prognostic markers specifically meant for breast cancer. This study also focuses on highlighting genes that are highly or lowly expressed in breast cancer which will help in understanding the diagnosis, progress, and development of personalized therapies for breast cancer.

2. MATERIALS AND METHODS

In order to identify meta-analysis of BRCA1, BRCA2, BRCA1/2 and normal genes involved in breast cancer of both familial, sporadic, familial cancer aggregation and normal cells that help the role of gene expression in disease progression can be analyzed using raw microarray data. We need to determine the disease mechanism and the role of signaling pathways, (i) Gene expression measurements (ii) definitions of signaling pathways and (iii) drug target identification.

2.1 Raw data selection

We searched public gene expression datasets from GEO microarray database to identify expression patterns neo-adjusted chemotherapy patients seriously affected gene expression patterns and profiles will identify used 3 different conditions. First 12 samples of breast cancer affected to women with hereditary BRCA1 gene mutation, 1 sample from BRCA2 gene mutation of hereditary women, 8 samples had BRCAx (BRACA1/2) type mutation of familial history of breast/ ovarian cancer, 10 samples were sporadic disease, 4 samples are familial cancer aggregation (FCA) but without prevalence of breast/ovarian cancer and 6 normal breast samples. The entire probe sets are annotated with REFERENCES platforms of hgu133plus2 is used to compare the gene names to identify differentially expressed genes (GSE50567) [20]. The overall flowchart is mentioned in Figure:1.

2.2 Preprocessing of raw data

To make a quality of data to identify differentially expressed genes in breast cancer using different pre-processing techniques such as (a) filtering data of image intensity values (b) remove bias using null values filtering and normalization, background adjustment, and gene transformation (c) quality control. There are different computational algorithms such as RMA, MAS5, GCRMA, and Li-Wong of R and Bioconductor statistical packages to predict normalization. The RMA algorithm creates expression matrix of raw intensity values of both foreground/background models corrected with the log of the linear transformation. The

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MAS5 algorithm normalizes each array independently and sequentially to predict the local average of mismatch and perfect match of background intensity corrections. The GCRMA algorithm helps to adjust for background intensities in Affymetrix array data which include optical noise and non-specific binding data. The Li-Wong algorithm originally calculated PM-MM calculation that suggests noise in all the probe measurements of roughly same size. Another Bioconductor package such as Biobase is used to create expression sets of Affymetrix datasets that used to develop phenotype data (pData). Different data normalization visualization platforms to predict box plots, density plots, PCA plots that help for normalization prediction of raw data.

2.3 Differential Expression Analysis

The limma package of Affymetrix data is used to identify differentially expressed genes in breast cancer. The simplest dataset features replicated designs and progress through experiments with two or more groups, direct designs and factorial designs of different time course experiments. To assign column names of expression set creates contrast matrix to perform all pair wise comparisons to compute estimated coefficients and standard errors of a given datasets. Computes moderated t-statistics and log-odds of differential expression by empirical Bayes shrinkage of the standard errors towards a common value. Generates list of top 10 ('number=10') differentially expressed genes sorted by B-values ('sort.by=B') for each of the three comparison groups ('coef=1') in this sample set. The summary table has logFC is the log2-fold change, the AveExpr is the average of expression value across all arrays and channels, the moderated t-statistic (t) is the logFC to its standard error, the P.Value is the associated p-value, the adj.P.Value is the p-value adjusted for multiple testing and the B-value (B) is the log-odds that a gene is differentially expressed (the-higher-the-better). Usually one wants to base gene selection on the adjusted P-value rather than the t-value or B-values. Filters out candidates that have P-values < 0.05 in each group ('coef=1') and provides the number of candidates for each list. These numbers should be identical with the sum of the values in each circle of the above Venn diagram. Same as above, but with complex filter: P-value < 0.01 AND at least 2-fold change AND expression value A > 10. This function plots, heat diagram gene expression profiles of genes which are significantly differentially expressed in the primary condition (this is not a cluster analysis heat map). Genes are sorted by differential expression under the primary condition. The argument 'primary=1' selects the first contrast column in the 'results' matrix as a primary condition.

2.4 Meta-analysis and classification

Using geNETClassifier algorithm to classify the genes was differentially expressed in different disease datasets along with gene networks. The genome-wide association studies of expression sets or expression matrix files of ranked genes, probe sets of different variables are optimized with training sets. Using multi-class SVM based classifier to quires genes chosen for classification; the mutual-information (interactions) and the co-expression (correlations) between the genes are also calculated and analyzed by the algorithm. These allow estimating the degree of association between the variables and they are used to generate a gene network for each class. These networks can be plotted, providing an integrated overview of the genes that characterized each disease (i.e. each class).

2.5 Functional Annotation and Enrichment Analysis

In order to obtain the functional enrichment of the differentially expressed genes on the cell level, we used the GO (Gene Ontology) database to classify the gene function and location information. We performed GO cluster analysis by using the cluster Profiler package, then deduced the affection of these differentially expressed genes to the cells by cluster the cells within the molecular functions and biological processes. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov) and GOrilla

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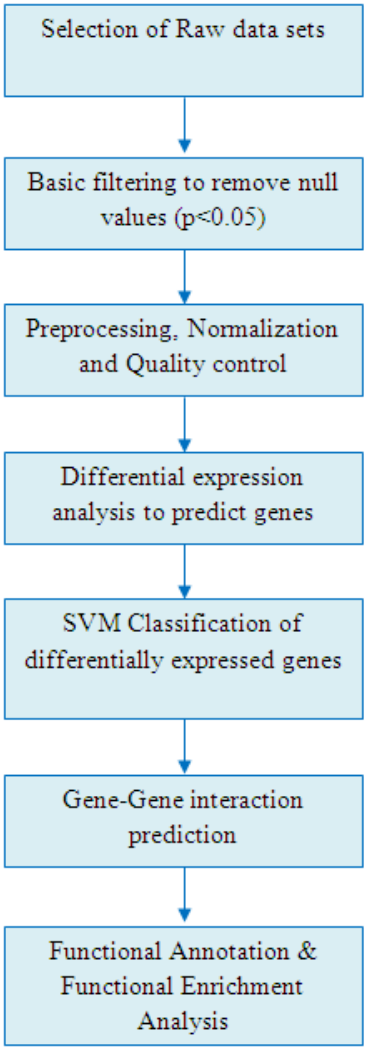
tool were used to identify over-represented biological functions and pathways among the differentially expressed genes.

Figure: 1. A flowchart for the microarray data analysis of breast cancer data.



3. RESULTS AND DISCUSSION

In the current study, we have predicted the analysis of breast cancer genes that are classified based on familial, sporadic, FAC and normal cell types. Using Meta-analysis techniques to classify differential expression of genes involved in breast cancer of BRCA1, BRCA2, and BRCA1/2 mutations. There are different sets of genes while comparing the hereditary (BRCA1-BRCA2), BRCA1-BRCAx, BRCA1-Sporadic, BRCA1-FCA, BRCA1-Normal, BRCA2-BRCAx, BRCA2-sporadic, BRCA2-FCA, BRCA2-normal, BRCAx-sporadic, BRCAx-FCA, BRCAx-normal, sporadic-FCA, sporadic-normal, FCA-normal sets to classify up-regulated and down-regulated genes that functionally involved in breast cancer.

3.1 Differential gene expression analysis

After normalization of raw data by calculating foreground and background intensities of a color of all probe sets is used to predict differential gene expression. Using limma package to determine the differential gene expression based on significance prediction of p-value (<0.05). A total of 54675 genes that significantly used for differential classification, A total of 592 genes is up-regulated and 810 genes show down-regulated that differentially expressed in

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BRCA1 gene mutation of ER signaling pathways in breast cancer. Using hierarchical clustering of 41 datasets using the 810 genes out of 54675 genes with unique gene resulting in the main branch, it is sub-classified according to the type of samples hereditary BRCA1, hereditary BRCA2, hereditary BRCAx, Sporadic, Sporadic FCA and Normal cell types by PAM50 classifier. The BRCA1 related most of the samples is cancers in ER cluster including BRCA1, BRCA2, BRCAx, FCA tumors of breast cancer (Table:1a; 1b; 1c; 1d) (Figure 2).

Table: 1a Differential expression of BRCA1 gene mutation in ER cells of breast cancer

BRCA1 - BRCA2		BRCA1- BRCAx		BRCA1- Sporadic		BRCA1 – FCA		BRCA1- Normal	
Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
86	82	22	40	27	48	20	42	331	369

Table: 1b Differential expression of BRCA2 gene mutation in ER cells of breast cancer

BRCA2 – BRCAx		BRCA2- Sporadic		BRCA2 –FCA		BRCA2 -Normal	
Up	**Down**	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**
48	72	36	58	21	162	25	71

Table: 1c Differential expression of BRCAx gene mutation in ER cells of Breast cancer

BRCAx – Sporadic		BRCAx- FCA		BRCA2 –Normal	
Up	**Down**	**Up**	**Down**	**Up**	**Down**
56	78	66	58	29	62

Table: 1d. Differential expression of FCA and Normal cells in ER cells of breast cancer

Sporadic-FCA		Sporadic- Normal		FCA - Normal	
Up	**Down**	**Up**	**Down**	**Up**	**Down**
18	98	1554	771	42	46

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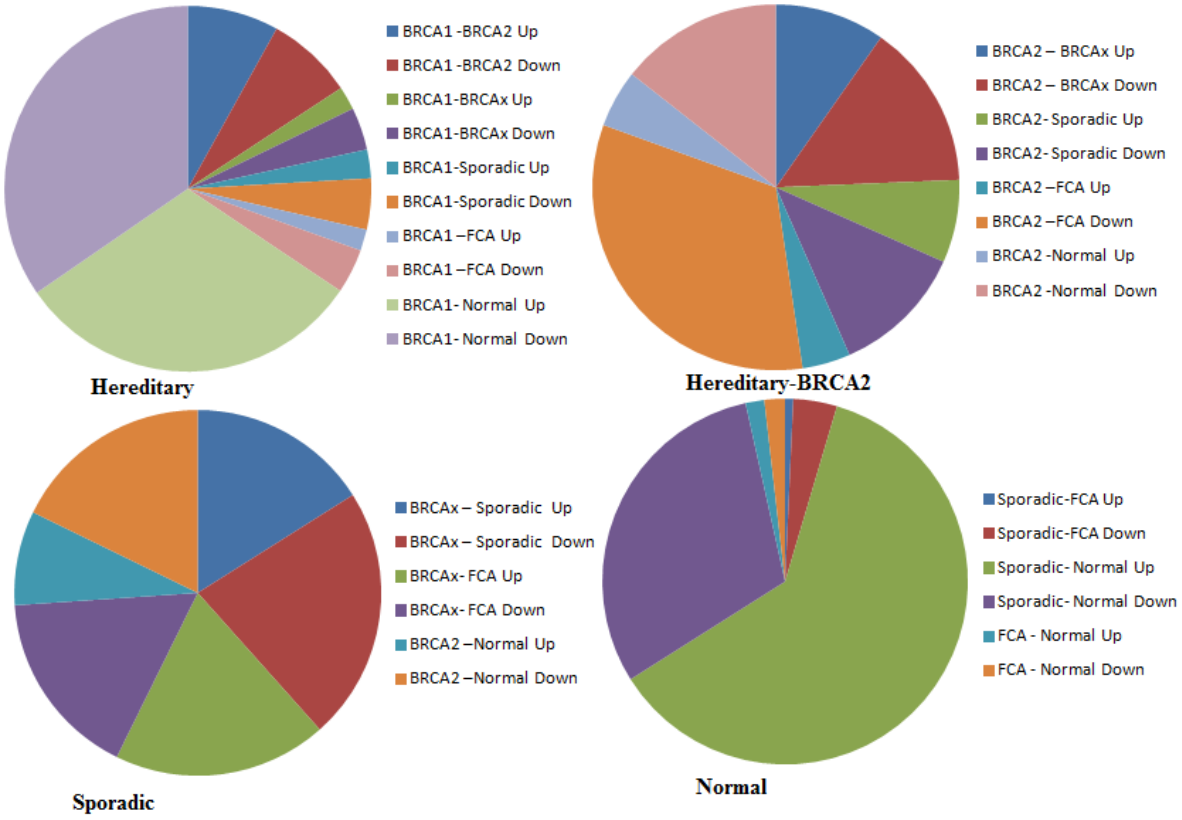
Figure: 2. Differential gene expression of cancer genes (P<0.01)



3.2 Molecular subtypes of BRCA1 mutation related genes in breast cancer

The RNA expression profiling of BRCA1 mutations in breast cancer that carries a general classification of hereditary, sporadic and normal cells. All the subgroups of BRCA1 show KCJN3, CDH2, Bnc1 is more significantly associated with all the subgroups. Using clinical consideration of all top 10 up-regulated and down-regulated genes show KCJN3, CDH2, LOC645323, RCBTB1, CYP27C1, Bnc1, and FXC1 is the significant association with BRCA1 gene transcriptional regulation and cause mutations. The other genes such as KCNJ3, MYST4, DSP, SLC6A, and EDDM3B is mainly associated with the BRCA1 gene mutation, using functional enrichment analysis shows 19 genes encode for protein synthesis that significantly associated with transcriptional regulation control and cellular response of apoptosis. The overall list of genes is predicted in the Table: 2.

Table: 2. Up-regulated genes of BRCA1 genes predicted based on p<0.01

ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
KCNJ3	-0.931303	2.064291	-13.7946	9.28E-14	5.08E-09	13.6160
KCNJ3	-1.462971	2.060524	-8.60714	3.14E-09	8.59E-05	8.14517
MYST4	-0.37652	1.937089	-8.07720	1.10E-08	0.000157	7.35611
DSP	0.529532	3.556083	8.057476	1.15E-08	0.000157	7.32576
SLC6A4	-0.50335	1.884686	-7.84098	1.95E-08	0.000213	6.98823

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KCNJ3	-0.81537	2.291112	-7.44479	5.16E-08	0.000470	6.34862
EDDM3B	-0.51841	1.974014	-7.27400	7.90E-08	0.000616	6.06417
SLC14A2	-0.46968	1.846501	-7.06218	1.35E-07	0.000894	5.70415
Six4	-0.56605	1.809206	-7.02631	1.47E-07	0.000894	5.64239
aqp11	-0.46806	2.514479	-6.54194	5.09E-07	0.002783	4.78648

3.3 Differential classification of BRCA2 mutation related genes in Breast cancer

The BRCA2 mutation related gene classification shows 22 up regulated and 40 down regulated genes that differentially expressed in BRCA2 gene mutation in ER cells of breast cancer. The top 10 genes such as Mir9-2 is post-transcriptional regulation of gene expression in multicellular organisms affecting both the stability and translation of mRNAs. The CDH2 receptor encodes protein receptor of calcium-dependent cell-cell adhesion of glycoprotein comprised of five extracellular cadherin repeats of transmembrane regions that have highly conserved in the cytoplasm tail is mainly significant genes in BRCA2 mutation in breast cancer (Table: 3).

Table: 3. Up-regulated genes of BRCA2 genes predicted based on p<0.01

ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
Mir9-2	0.6957603	2.220272	7.6294039	3.27E-08	0.0017874	5.68855
CDH2	0.4915699	2.139586	6.6502505	3.85E-07	0.0105239	4.22686
GLC	0.4155692	2.000924	5.6926966	4.74E-06	0.0863581	2.63784
Bnc1	0.7709860	2.027141	5.4618724	8.77E-06	0.1017289	2.23363
RCBTB1	-0.425034	2.884555	-5.439906	9.30E-06	0.1017289	2.19479
Bnc1	0.8099578	1.977054	5.2163559	1.69E-05	0.1543520	1.79598
NPL	0.1634098	2.141903	4.9712574	3.27E-05	0.2556709	1.35210
SHROOM2	-0.281433	3.023001	-4.912555	3.83E-05	0.2619858	1.24488
CDH2	0.2152811	2.780284	4.856113	4.46E-05	0.2710691	1.14149
FXC1	-0.191883	3.117282	-4.815805	4.97E-05	0.2719095	1.06749

3.4 Differential expression of BRCAx samples in breast cancer

The BRCAx is compared with sporadic samples of breast cancer shows 27 up regulated and 48 down-regulated genes. The 10 top regulated genes such as CDH2 is a common regulator of cell-cell adhesion in transmembrane protein, the LOC645323 gene has transcriptional regulation in putative alternative promoters of breast cancer genes, and RCBTB1 interacts with the ACE2A receptor that regulates B-cell (Table: 4).

Table: 4. Up-regulated genes of BRCAx genes predicted based on p<0.01

ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
CDH2	0.511196	2.139598	7.058374	1.36E-07	0.004731	4.96266
LOC645323	0.622137	2.220272	6.962767	1.73E-07	0.004731	4.81529
RCBTB1	-0.45930	2.884555	-5.99974	2.10E-06	0.024058	3.24239
CYP27C1	0.820491	2.027141	5.932442	2.51E-06	0.024058	3.12672
CDH2	0.256893	2.780284	5.914256	2.63E-06	0.024058	3.09534
Bnc1	0.899597	1.977054	5.913128	2.64E-06	0.024058	3.09340
GLS	0.398676	2.000924	5.573903	6.50E-06	0.050793	2.49901
FXC1	-0.20846	3.117282	-5.33986	1.22E-05	0.083114	2.07951
EBPL	-0.32946	3.380571	-5.18841	1.83E-05	0.110920	1.80451
Bnc1	0.395175	2.332388	5.072930	2.49E-05	0.133939	1.59303

3.5 Differential expression of sporadic gene mutation samples in breast cancer

In Sporadic gene mutation shows 18 up-regulated genes and 98 down-regulated genes that differentially expressed in sporadic genes. To evaluate the reproducibility of the expression

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patterns of the signatures shows LOC645323, CDH2, CYP27C1, RCBTB1 genes is significantly associated with transcriptional regulation within metastasis (Table: 5). This revealed 18 Sporadic- 98 sporadic basal-like tumors in the RCBTB1 data set. The performance of the signature was estimated by LOC645323, using the SVM algorithm.

Table: 5. Up-regulated genes of sporadic genes predicted based on p<0.01

ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
LOC645323	0.710737	2.220272	7.114588	1.18E-07	0.006443	4.437710
CDH2	0.549862	2.139598	6.790722	2.68E-07	0.007337	3.980876
CYP27C1	0.906285	2.027141	5.860969	3.03E-06	0.041897	2.569314
RCBTB1	-0.50127	2.884555	-5.85673	3.07E-06	0.041897	2.562568
GLS	0.436698	2.00092	5.460915	8.79E-06	0.096169	1.919413
Bnc1	0.874186	1.977054	5.13946	2.08E-05	0.189758	1.381185
CDH2	0.241086	2.780284	4.96437	3.33E-05	0.260447	1.082783
Tmem111	-0.27922	3.087107	-4.80371	5.14E-05	0.351129	0.806166
FAM134A	-0.18538	3.28670	-4.74348	6.04E-05	0.366654	0.702488
IL23A	0.268365	2.347062	4.599524	8.90E-05	0.475319	0.451335

3.6 Functional enrichment

Based on functional annotation and enrichment analysis of familial, sporadic, BRCA1, BRCA2, BRCAx, FCA of both gene sets and functional categories can influence the results of functional enrichment analysis. In order to mitigate these effects, we suggest that instead of evaluating the overlap between 50 genes such as KCNJ3, MYST4, DSP, SLC6A4, EDDM3B, Six4, aqp11, Mir9-2, CDH2, GLC, Bnc1, RCBTB1, NPL and SHROOM2 gens traditionally done in functional enrichment analysis, one instead considers the overlap between the annotations made to a gene set and a branch of terms in the Gene Ontology. To accurately capture the significance of annotation overlap we develop a randomization scheme that preserves the transitive annotation features of the GO DAG while calculating the probability of obtaining a certain number of annotations between a gene set and a GO branch (Table: 6).

Table: 6. Top genes that significantly associated with breast cancer

Genes	p-value	q-value Bonferroni	q-value FDR B&H	q-value FDR B&Y
GATA3,FOXA1	5.15E-07	1.51E-03	3.78E-04	3.24E-03
FOXA1,TOX3	5.15E-07	1.51E-03	3.78E-04	3.24E-03
AGR3,AGR2	5.15E-07	1.51E-03	3.78E-04	3.24E-03
MMP12,PTX3	5.15E-07	1.51E-03	3.78E-04	3.24E-03
GATA3,FOXA1	1.54E-06	4.54E-03	4.54E-04	3.88E-03
AGR3,AGR2	3.09E-06	9.07E-03	6.98E-04	5.97E-03
GATA3,FOXA1	3.09E-06	9.07E-03	6.98E-04	5.97E-03
GATA3,TOX3	2.07E-04	6.07E-01	2.47E-03	2.12E-02

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AREG	7.34E-04	2.16E+00	2.47E-03	2.12E-02
GATA3	7.34E-04	2.16E+00	2.47E-03	2.12E-02
SCGB2A2	7.34E-04	2.16E+00	2.47E-03	2.12E-02
ANKRD30A	7.34E-04	2.16E+00	2.47E-03	2.12E-02
FOXA1	7.34E-04	2.16E+00	2.47E-03	2.12E-02
GATA3	7.34E-04	2.16E+00	2.47E-03	2.12E-02
VGLL1	7.34E-04	2.16E+00	2.47E-03	2.12E-02
MMP12	7.34E-04	2.16E+00	2.47E-03	2.12E-02
TOX3	7.34E-04	2.16E+00	2.47E-03	2.12E-02
ANKRD30A	7.34E-04	2.16E+00	2.47E-03	2.12E-02
AREG	7.34E-04	2.16E+00	2.47E-03	2.12E-02

3.7 Significant analysis of breast cancer genes in biomarkers

Using SVM classification technique, we have classified 810 genes is differentially expressed in four conditions such as familial expression of BRCA1, BRCA2, BRCAx, Sporadic, FCA and normal cell types. Further, we have classified the genes based on functional enrichment shows only 592 genes encode proteins; this encoding protein is a significant expression of breast cancer. We have separated the genes which only expressed in breast cancer shows only 30 genes are the most common expression in breast cancer and is potentially used as biomarkers. The most common genes such as GATA3, Foxa1, Tox3, Arg3, Arg2,Mmp12, Ptx3, AREG, SYNPO2, Scgb2a2, TFAP2B, Ptx3, Dach1, Vgll1, REEP6, MPP7, ANKRD30A, Igf2bp3, CYP4Z1, SLC6A4, FAM134A, DSP, PSPH, SDR16C5, AQP11, GLS, PSPH, TMC5 and SLITRK6 genes is mainly associated with transcriptional regulation (Table:7; Figure: 3).

Figure: 3. Breast cancer associated genes predicted using an SVM data

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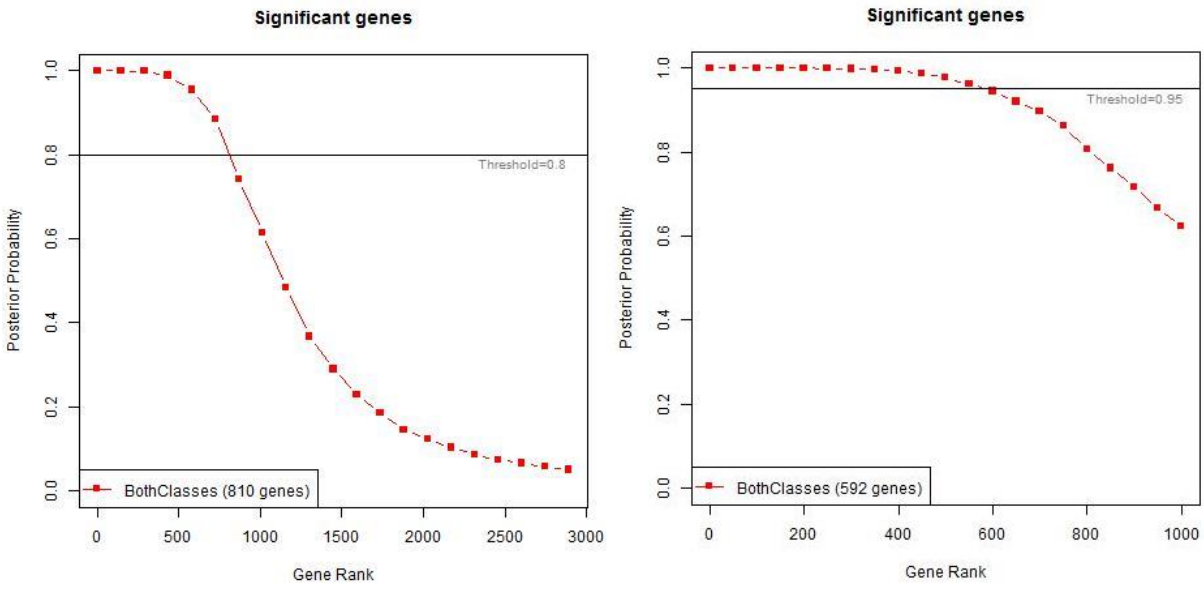

Table: 7. Gene ranking of SVM classified gene signatures predicted using Breast cancer data

Gene Ranking	Genes	MeanDiff	Expression Up/Down	Redundancy
1	VGLL1	1.2714	UP	FALSE
2	REEP6	-1.2938	DOWN	FALSE
3	SYNPO2	1.1423	UP	FALSE
4	AREG	-1.1253	DOWN	FALSE
5	LYZ	1.1246	UP	FALSE
6	TOX3	1.1043	UP	FALSE
7	TOX3	-0.8966	DOWN	FALSE
8	TOX3	1.0233	UP	TRUE
9	AGR3	0.997	UP	FALSE
10	PTX3	1.027	UP	FALSE
11	MPP7	1.0024	UP	TRUE
12	DACH1	0.8899	UP	TRUE
13	DACH1	0.9032	UP	TRUE
14	TFAP2B	0.8417	UP	TRUE
15	TFAP2B	0.8214	UP	FALSE
16	ANKRD30A	0.8232	UP	TRUE
17	MMP12	0.7949	UP	TRUE
18	GATA3	0.7965	UP	TRUE
19	PSPH	0.8386	UP	FALSE
20	SCGB2A2	0.8309	UP	TRUE
21	SLITRK6	0.767	UP	TRUE
22	LINC00993	-0.7531	DOWN	FALSE
23	AGR2	0.7077	UP	FALSE
24	IGF2BP3	0.7999	UP	TRUE
25	SDR16C5	-0.8356	DOWN	FALSE
26	CYP4Z1	0.7044	UP	TRUE
27	TMC5	0.7001	UP	FALSE

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28	TMC5	0.7613	UP	FALSE
29	FOXA1	0.7132	UP	TRUE
30	FOXA1	0.7184	UP	TRUE

3.8 Gene network prediction

We used SVM method to construct the network using a geNETClassifier algorithm to identify informative gene pairs and assign weights to sample pairs. There are two parameters to combine optimal combinations such as accuracy of the k-model fold cross validation by varying threshold values from 0.11 to 0.8 in intervals of 0.01 and another threshold value of 0.85 to 0.9 in intervals of 0.5. We have performed 26 different experiments that varying these two threshold values that measure the accuracy of the gene network. There are 592 genes were involved in a gene network that has a significant association with 54 genes alone have optimal isolation. The overall gene network is predicted in Figure: 3 & 4.

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Figure: 3. Gene-gene network predicted using geNETClassifier for significantly expressed breast cancer genes

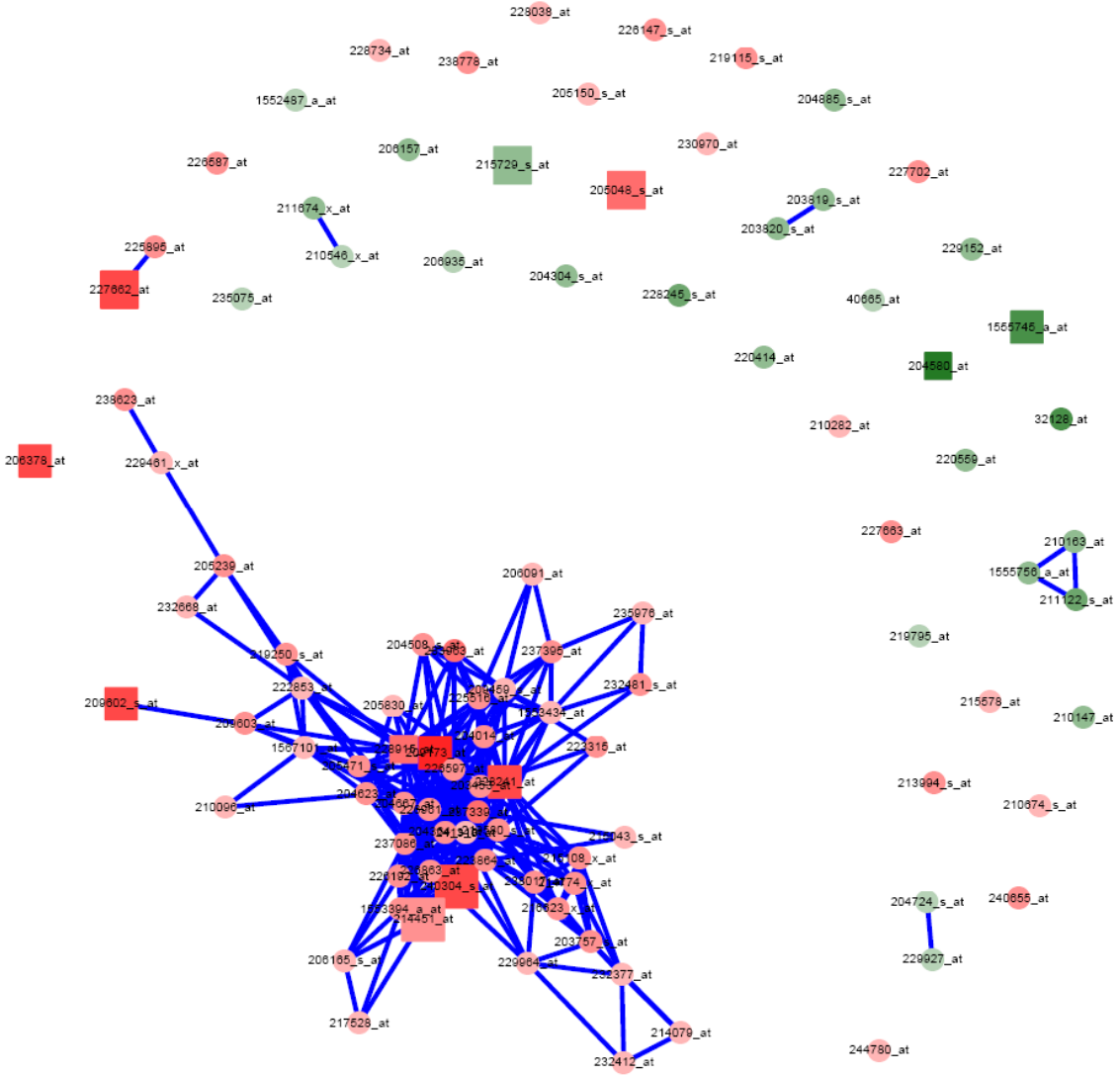

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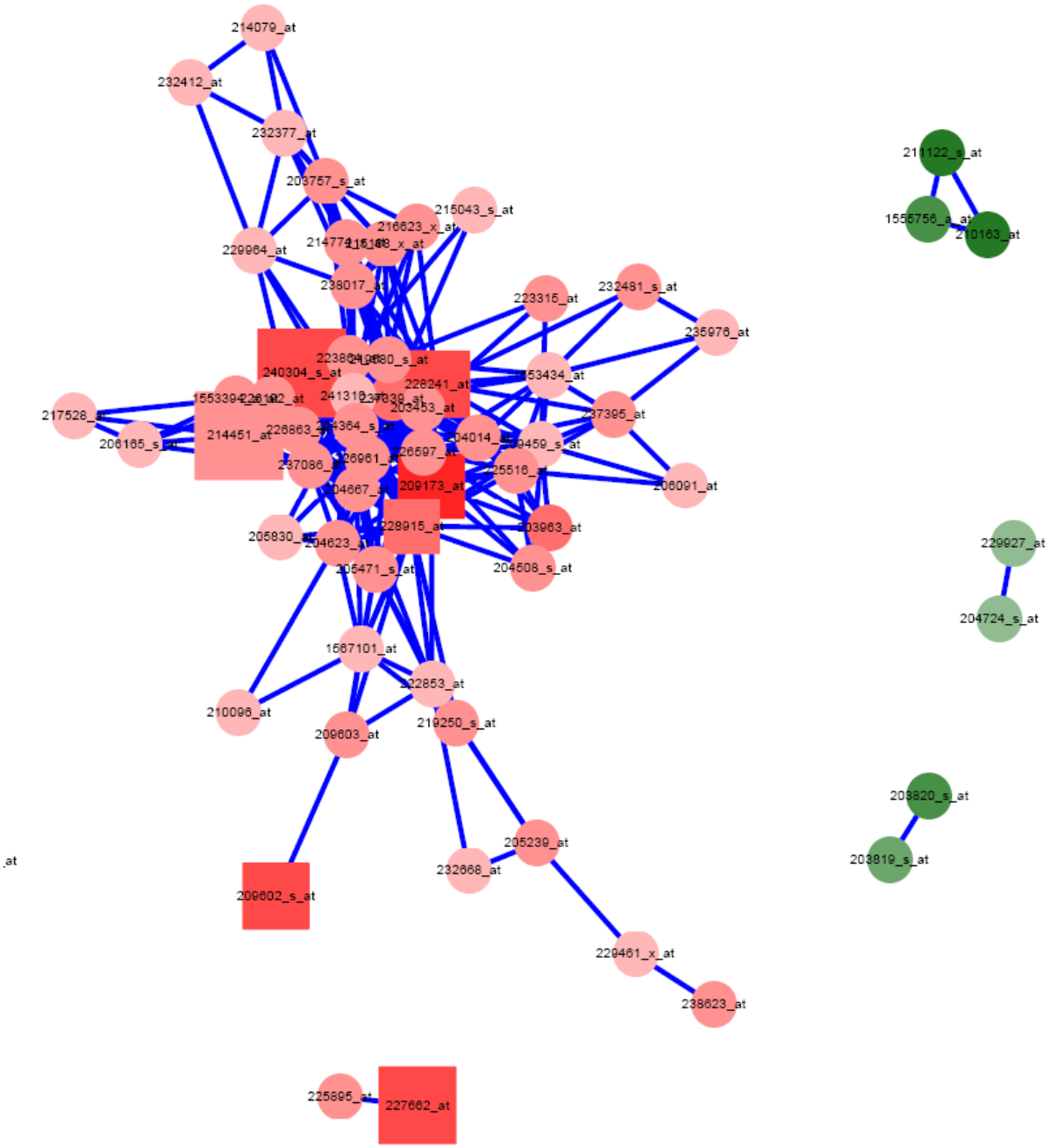
Figure: 4. Top30 gene signatures predicted using the SVM Classifiers



4. CONCLUSION

In the current study of developing Meta-analysis of Identification of BRCA1 mutated gene signatures present in breast cancer is presented. There are 6 types of datasets such as familial of BRCA1, BRCA2, BRCAx, Sporadic, FCA and normal sets of specific gene signatures. We have identified 810 genes that are significantly associated with all the types of datasets. However, 592 genes are linked to the metastatic condition. There are 52 genes involved in different pathways associated with metastasis. We have compared the list of genes in 6 independent groups and demonstrated that 30 genes are enriched for GO annotation that

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significantly associated with different pathways of all 6 types of datasets and these genes are mainly used for potential drug targets. Furthermore, these results help to identify metastatic signature to facilitate further research in metastasis.

CONFLICT OF INTEREST

There is no conflict of interest

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DIFFERENTIALLY EXPRESSED NEUROGENIN-3 GENE IN NEURONAL CELL DEVELOPMENT OF PANCREATIC CELL

Sonika Rai, Alivia Saha, Ragini V Murthy[2], Ramachandra R[1], Prashantha Nagaraja[1]
[1]Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560064
[2]Department of Biotechnology, Dayanad Sagar College of Engineering, Bangalore.

ABSTRACT: Neurogenin3 is a family of basic helix-loop-helix (bHLH) transcriptional protein involved in endocrine development in pancreas and intestine. The bHLH regulates transcription of neurogenin3 (ngn3) of progenitor ductal and endocrine cells to a neurogenesis and neurogen specific transcriptional mechanisms that mediates different signaling pathways. The differentiated genes such as Pdx1, Ngn3, Pax6, Rpf6, Isl1 and Nkx2.2 genes regulating the development of islet of endocrine and ductal cells, but the molecular mechanism and classification of gene expression is remain unknown. There are several transcriptional gene mutations may regulate transcription of islet cells of ductal and endocrine regions of the pancreas and intestine that may lead to cell. However, our knowledge of microarray data analysis methods helps to classify the genes associated with differential and undifferential endocrine lineage, ductal cell and exocrine regions determine neurogenesis and neuron specific signaling pathways. Using meta analysis of statistical rank correlation algorithm to rank the genes based on gene signatures. The reveal predicts 154 (38%) genes that were consistently and significantly upregulated, 247 (62%) were downregulated in pancreatic cell. Using functional annotation of gene clusters reveals only 47 genes is presumably associated with neuronal development and cell differentiation. Furthermore, this experiment helps to understand candidate genes for novel biomarkers identification, diagnosis and therapeutic approaches to pancreatic cell.

KEYWORDS: Pancreatic cell, Ngn3, gene expression, Meta Analysis, Microarray, Neurogenin, Neuronal development

***Corresponding Author:** Dr. C. N. Prashantha
Department of Biotechnology, School of Applied Sciences, REVA University, Bangalore-560092, Karnataka, India
E-Mail: prashanthacn@gmail.com;

1. INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer deaths [1] among men and women, being responsible for 6% of all cancer-related deaths and it is very difficult to diagnose in its early stages. At the time of diagnosis, 52% of all patients have distant disease, 26% have survival rate, past 5 years only 21.5% of survival rate has been recorded [2]. With non-specific symptoms 90% of patients are diagnosed for surgery in advanced stage. Prior to this study, nearly 95% of PC is associated with hereditary factors that may greater risk of PC [3]. The lethal nature of PC stems from its propensity to rapidly disseminate to the lymphatic system and distant organs. There are various types of pancreatic cancer include 80% are adenocarcinomas of the ductal epithelium. Only a pair of of tumors of the exocrine duct gland are benign.

In most patients, the PC is observed in both exocrine and endocrine regions, however the biological phenomenon shows the exocrine region release insulin-producing beta cells during embryogensis and endocrine region regulates glucose tolerance [4]. The pathological association of diabetes in exocrine region and pancreatic cancer is in the endocrine region

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[5,6]. During the development of progenitor cells, which express specific genes Foxa1, Foxa2, Pdx1, Pbx1, Hes1, Ptf1a, Ngn3, HNF6, Pax4, NeuroD1, Nkx2.2 and Sox9, give rise to express in both the ductal, exocrine and endocrine pancreatic cell development [7]. In cell division, the inactivation of genes that may transcriptionally regulate embryogenesis of neuronal cells it may leades to cancer [8]. The bHLH transcriptional factors Neurog3 (ngn3) controls a complex of gene regulatory transcriptional networks in endocrine progenitor cells which regulate insulin secreting islets of Langerhans [9]. The relationship of exocrine and endocrine development of multipotent intracellular genes that may limit the expression during precursors of ductal polypeptide producing cells.

The experimental results suggests that the insulin producing genes such as Isl1, PDX1, neuroD1 (BETA2), glucogon promoter genes Pax6, cdx2/3, Foxa1, Foxa2, HNF3α and β is differentially expressed in Notch and Hedgehog pathways [10]. Although there are other transcriptional signaling pathways play important regulatory roles during PC development and the molecular mechanism are unknown. There are various techniques to understand the molecular mechanism and role of genes in intracellular signaling pathways. Using gene expression program determines the characterization of Ngn3-mediated duct and endocrine cell mediated signaling pathways. The studies generated large set of adenoviruses expressing Ngn3 and GFP (AdGFP-Ngn3) or GFP only (AdGFP) of genome wide mRNA profiling data is revealed the expression analysis to reprogram ngn3 expression on pancreatic cell gene analysis [11].

The objective of the study is to understand the disease ethiology of novel candidate gene expression in disease progression, cell cycle, neuronal development and cell differentiation. We used gene expression dataset to understand the characterstic of ngn-3 mediated mRNA profiling within duct, exocrine and endocrine cell functional genes that may involved in PC and diabetes mellitus. We evaluate the transcriptional regulation of genes that may involved in expression with β-islets within endoderm, that may helps to synthesize insulin, neuronal development mediated gene signaling networks that may helps to synthesize neuronal cells to form interconnection between pancrease and intestine. In this we temporarly targets pdx1 and ngn3 induced pancreatic with β-islets within endocrine cells, the genes that differentially expressed in both drug targets and disease conditions are typically predicts for molecular biomarkers. The biomarkers helps to identify the genes associated with neuronal cell development of pancreas along with other types of diseases also.

2. MATERIALS AND METHODS

2.1 Disease dataset selection

To identify $Ngn3^{+}$-mediated transcriptional regulation of pancreatic endocrine islet cells mediated positive regulation and associated gene signaling pathways is predicted using whole mRNA microarray datasets (GSE30802) [11]. There are 12 datasets contains 6 adGFP control vectors and 6 adGFP-Ngn3 mediated samples to differentiate expression profiles of genes which is involved in transcriptional regulation within pancreatic cell development. Each dataset contains 22645 probes of different intensity values are annotated with Affymetrix human genome U133B array (hgu133b). The REFERENCES annotation file contains 44928 gene entries is selected from GenBank, dbEST and RefSeq databases is refines and compared with selected datasets. Using R and BioConductor to validate the datasets using pre-processing, normalization and differential expression profiling using different algorithms. The advantage of using this data is to understand the expression of ngn3 mediated human duct cells is recombinant with adenovirus and the induction of ngn3 expressions with different time intervals. The different time intervals is helps to predict the diffferntial

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expression of ngn3 in duct, endocrine and exocrine cell differentiation in pancreas.

2.2 Preprocessing and Normalization

The dataset is from a gene expression levels of pancreatic samples from H.sapiens, the details of study is given in Martens GA et.al (2012). The dataset contains 6 controls of human ductal cells is transfected with AdGFP control vector biological samples and 6 treated human duct cell after transduction with Ad-Ngn3-GFP vectors in 3, 14 and 20 days time series. The samples is labeled with Cy3 (green dye) of control and Cy5 (red dye) of treated samples is hybridized with Affymetrix HG133B samples. However, the preprocessing and normalization procedures is implemented using Bioconductor packages [12] to remove systematic variance and prepare datasets for further analysis.

The preprocessing of raw data using affy and limma packages to transform unbiased statistical metrics to predict quality of outlier data. The GCRMA (Gene-Chip Robust Multiarray Average) [13] algorithm include noise and non-specific binding (NSB) data calculation to optimize background intensities that adjust probe intensities to expression measurements and the normalized data is summarized using Robust Microarray Analysis (RMA) algorithm [14]. The raw signal intensities of randomly retained datasets are in position of the PM and MM for every probe pairs, the MM probes introduces more background noise on a raw intensity scale to correct for NSB. The log2 transformation of two-color arrays convert data to a linear scale value of each background corrected PM probe is obtained and these values are normalized using quantiles normalization.

M= log2(Cy5/Cy3)

A=log2(Cy5*Cy3)*0.5

The RMA and GCRMA packages helps for only PM signals of background corrections. Thus the normalization techniques used only for conjunction with (*pmonly)* PM correction method. We used Li-Wong procedure to normaliz arrays using invariant set of genes and then fits a parametric model to the probe set data [15].

2.3 Differential gene expression of ngn3 in Pancreatic data

After normalization and preprocessing the resultant data is used for statistical analysis to predict the differential gene expression. Using Limma package provides comparison of RNA targets designed based on empirical Bayesian methods. The linear models of each expression data is measured by log2 expressions of single channel platforms. The coefficients of the fitted models are designed by matrix of hybridized probe intensities results in differential expressions. The model matrix is designed based on conditions of each datasets of control and disease of both Cy3 and Cy5 datasets is empirically calculated by standard deviations. The overall computed empirical values are finally calculates t-statistics, moderated F-statistics and B-statistics (log2 intensities of differential expressions). We have imposed the log2 fold change of 1 measure the cutoff of p-value <0.05 that can predict in topTable shows absolute fold change of > 5 using each contrast co-efficient values that has differential expressed genes.

2.4 Cluster Analysis

Clustering algorithms such as hierarchical [16] and K-means clustering [17] or self-organizing maps (SOM)[18] used to generate partial solutions of single factors. Hiearchical clustering specifically describes the differential gene expression based on distance matrix are connected by a series of branches (clustering tree or dendrogram). This method helps to classify gene expression analysis of complete linkage outforms. K-means clustering algorithm predicts the best fitting between clusters and their representation using a predefined number of clusters. The prototype of randomly selected datasets of lowest dissimilarity and represents smallest cluster variance.

2.5 Gene-gene interaction prediction

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To predict multi-class of microarray data is measured by SVM classifier based on differential gene expression data using geNETClassifier algorithm. We have integrated machine learning and statistical methods to measure gene ranks with gene signatures, multiple class classifiers and gene network associations. Using multi-class SVM based classifier to quires genes chosen for classification; the mutual-information (interactions) and the co-expression (correlations) between the genes are also calculated and analyzed by the algorithm. These allow estimating the degree of association between the variables and they are used to generate a gene network for each class. These networks can be plotted, providing an integrated overview of the genes that characterized each disease (i.e. each class).

2.6 Evaluation of functional Annotation and Enrichment analysis

The clustering data of functional data is used for GO annotation of the genes extracted from Affymetrix HG133B annotation file. The Database for Annotation, Visualization and Integrated Discovery (DAVID) Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov) [19] used to identify list of genes associated with biological terms into organized classes. The organizes of significant genes shared specific terms by <5% of the genes paied with common GO terms that functionally related. Genome enrichement analysis is predicted using Gorilla (http://cbl-gorilla.cs.technion.ac.il/) [20] is a web based tool for identifying and visualizing enriched GO terms in ranked lists of genes, without requiring the user to provide explicit target and background sets. This is particularly useful in many typical cases where genomic data may be naturally represented as a ranked list of genes.

3. RESULTS AND DISCUSSION

3.1 Experimental analysis of Ngn3 over expression in pancreatic cell lines

The differentially expressed dataset contains 22645 genes, the preprocessing and normalization is carried out by RMA and MAS 5.0 algorithms to correct both background and foreground intensity values of both cy3 and cy5 color labeling of both cDNA and mRNA samples. Further to filter the unexpressed genes with lowest intensity ranges of log2 intensity values <0.05 has number low variants 14066 genes excluded 68 genes. Using GCRMA to predict normalization by quantile method to filter the overall gene values and the results shows only 8511 genes alone accepted with log2 intensity change and is further predicted in box plot and histograms.

We have made a matrix of different coefficients with different statistical p-values shows coef=1 of AdGFP-AdNGN3 with fold change of 1 (ifc=1) predicts only 53 genes is differentially expressed at empirical bayes t-statistical calculation, further, we have classified the same datasets with multiples of comparisions such as coef= 1 is AdGFP_3days – AdNGN3_3days, coef=2 is AdGFP_14days – AdNGN3_14days and coef=3 is AdGFP_20days – AdNGN3_20days classes is predicted based on fold change of 1 (ifc=1) (Table: 1).

Table:1. Pancreatic cancer datasets to predict the differential gene expressions in neuronal cell development

Coefficients (Coef)	Fold change (ifc)	Genes	Up	Down
Coef= 1 (AdGFP_3days – AdNGN3_3days)	1	120	38	82
Coef= 2 (AdGFP_14days – AdNGN3_14days)	1	705	175	530
Coef= 3 (AdGFP_20days – AdNGN3_20days)	1	49	19	26

We determine the differentiall expression of pancreatic cell differentiation genes were

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independently classified. Using fold change (FC) cutoff of 12 independent datasets and a FDR-corrected P-values (P<0.05) of 15410 genes were filtered from background corrections. Using functional enrichment and gene ontology to classified 11721 genes were differentially expressed in overall dataset. The Pearson's Correlation coefficient of hierarchical clustering shows the differentially expressed genes is aligned based on Euclidean distance. The highly significant Euclidean distance with poor correlations did not show statistically significance with correlations of series of outcomes (fig 2). A set of 400 differentially expressed genes of significant (P <0.05) thresholds of well defined clusters is used for linkage analysis (supplementary table:1).

3.2 Identification of upregulated genes

We used geNetClassifier to classify significant genes of differential expression at <0.95 threshold shows 182 upregulated, 229 downregulated. The initial screening is carried out with all pairwise z-vlues associated with significant correlation of upregulated and downregulated datasets. Using false positive prediction of 46 gene ranks were upregulated at two folds, 65 genes are also significantly expressed in precisely explained pancreatic cell (fig: 3a, 3b). Supplementary Table 1 provides the upregulated and downregulated genes have well established and significantly associated with lack of neuronal cell development in pancreatic cell development. Some well known genes include NPTX1, INSM1, NKX2-2, NEUROD1, and PCSK1 genes is down regulated in pancreatic duct, endocrine and exocrince cells and is less effective to synthesize neuronal cell development and differentiation into insulin production. Hence, these genes is potential targets to pancreatic cell. NPTX1, INSM1, NKX2-2, NEUROD1, and PCSK1 genes is significantly downregulated genes in pancreatic cell on neuronal cell development, but these genes directly associated with other types of cell such as breast cell, overian cell and other cell types.

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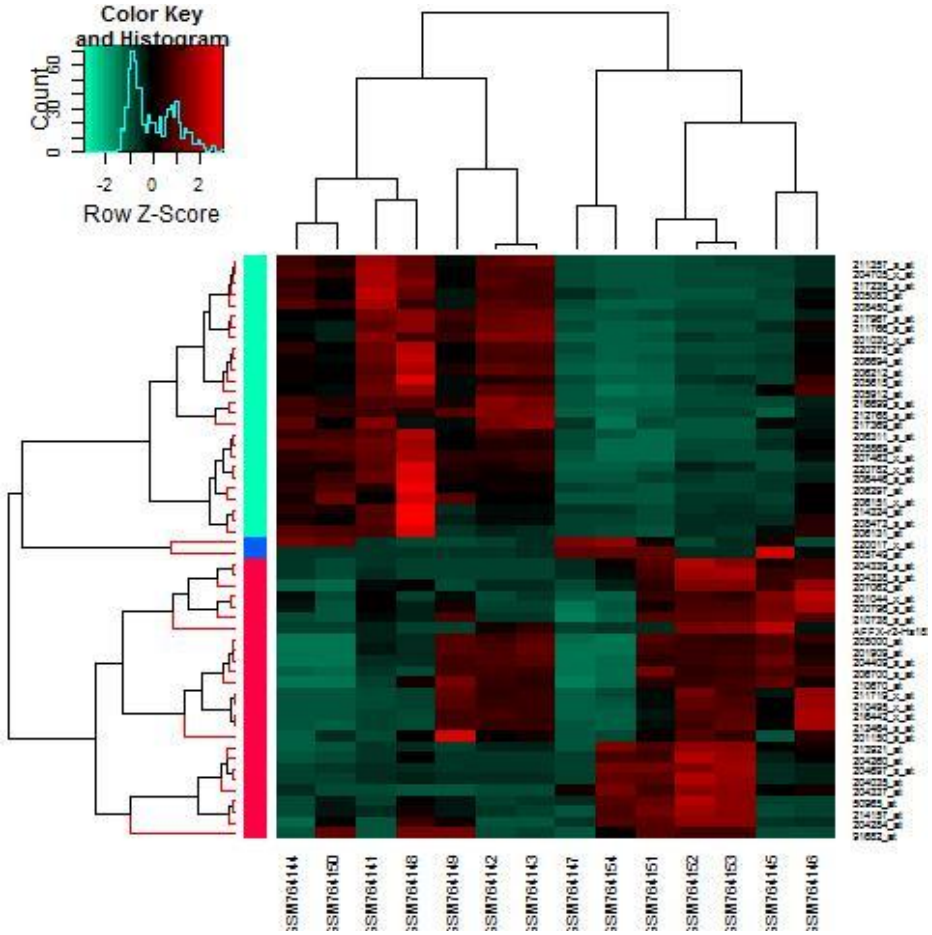

Fig: 2. Hierchical clustering of 400 differentially expressed significant genes predicted with p<0.05 threshold.

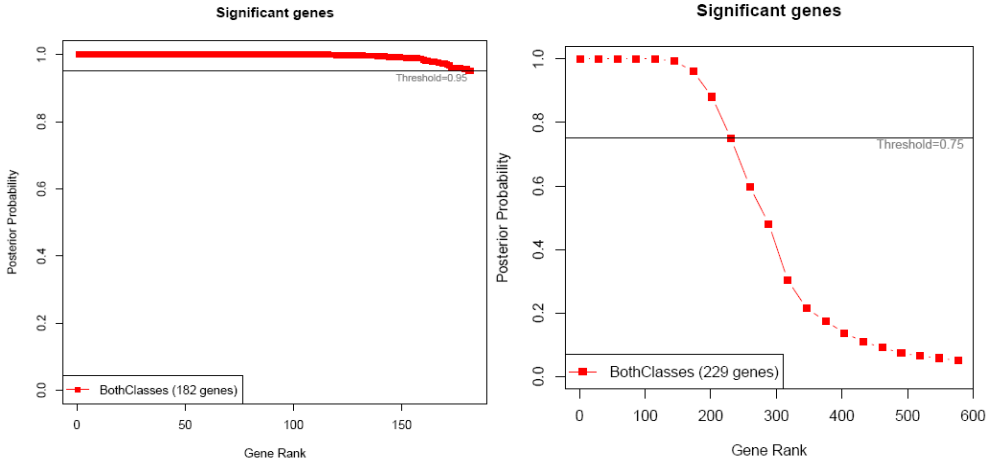

Fig: 3a Differential expression of case and control datase genes were predicted with significance of <0.05 threshold.

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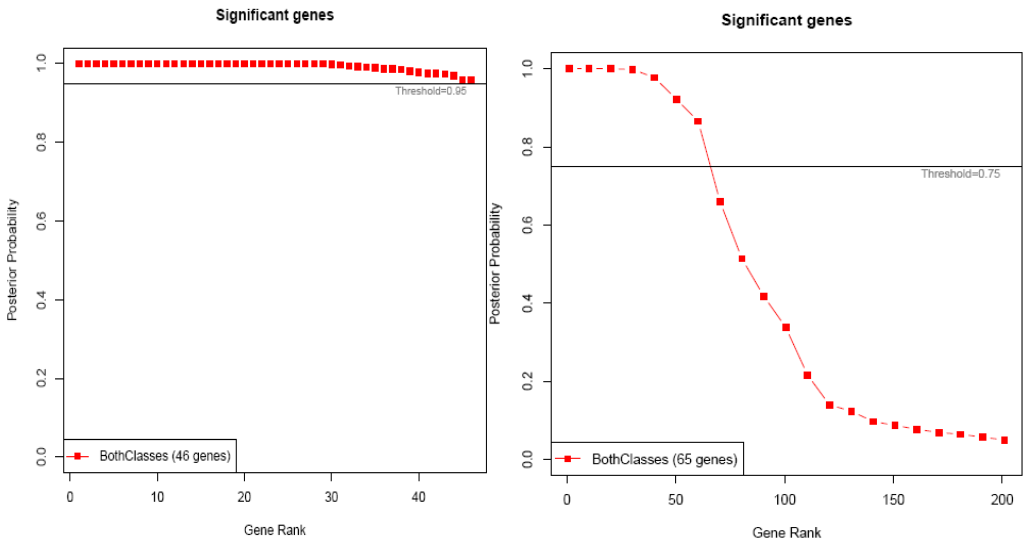

Figure 3b. Differential expression of both commonly associated biomarker genes is predicted with significance of <0.05 threshold.

3.3 Functional analysis of ngn3 associated significant genes

We identified the functions of 46 significant genes of both upregulated and downregulated genes were analysed using GOrilla online database. The most significant biological functions with 12 upregulated genes are functionally involved in cell-differntiation, cell development and disease progression with in pancreatic cells. The dysregulation of NEUROD1, NKX2-2 and NPTX1 of p-value 2.46E-5 is significantly involved in different pathways that integrated insulin secretion with in islet cells of pancreas and also involved in diabetes mellitus (supplementary table 3).

4. CONCLUSION

We have predicted the gene expression of ngn3 gene regulation in pancreatic neuronal cell development that leads to cell. The ductal, exocrine and endocrine cells of neurogenesis of expressed datasets predicts 46 genes of upregulated and downregulated that potentially signified in pancreatic cell and also associated with other significant cell types. Based on this analysis the genes NPTX1, INSM1, NKX2-2, NEUROD1, and PCSK1 is downregulated in pancreatic cells and also with different cell types is potentially predicted best biomarkers to understand pancreatic cell and associated cell types. Our results are helps to predict the gene regulation networks of other significant gene regulatory networks is helps to predict best drug targets and is used for drug discovery.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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Supplementary table: 1: Differentially significant genes of both classes of case and control studies with p<0.01

BothClasses.GeneName	BothClasses.ranking	BothClasses.postProb	BothClasses.exprsMeanDiff	BothClasses.exprsUpDw	BothClasses.isRedundant
NPTX1	1	1	-2.3004	DOWN	FALSE
INSM1	2	1	-2.2827	DOWN	TRUE
NKX2-2	3	1	-1.4654	DOWN	TRUE
NEUROD1	4	1	-1.2636	DOWN	FALSE
PCSK1	5	1	-1.1867	DOWN	TRUE
CHGA	6	1	-1.0649	DOWN	TRUE
PAEP	7	1	-0.9784	DOWN	TRUE
AC055876.5	8	1	-0.9432	DOWN	TRUE
SFTPA2	9	1	-0.8469	DOWN	TRUE
SCG2	10	1	-0.939	DOWN	TRUE
SCGN	11	1	-0.9178	DOWN	TRUE
RAB26	12	1	-0.8246	DOWN	TRUE
ENPP2	13	1	-0.8067	DOWN	TRUE
TAC3	14	1	-0.844	DOWN	TRUE
PCP4	15	1	-0.8117	DOWN	TRUE
CRYBA2	16	1	-0.8074	DOWN	TRUE
PNLIPRP2	17	1	0.917	UP	FALSE
SCG3	18	1	-0.7938	DOWN	TRUE
NEUROG3	19	1	-0.7728	DOWN	TRUE
GCH1	20	1	-0.7511	DOWN	TRUE
KRTAP9-5	21	1	-0.6746	DOWN	TRUE
NLRP1	22	1	-0.7249	DOWN	TRUE
PPP1R3C	23	1	-0.6434	DOWN	TRUE
ASCL1	24	1	-0.7412	DOWN	TRUE
UCP2	25	1	-0.6298	DOWN	TRUE
SPINK4	26	1	-0.4438	DOWN	FALSE
PAX6	27	1	-0.593	DOWN	TRUE
USP18	28	1	-0.5501	DOWN	TRUE
CHGB	29	1	-0.4924	DOWN	TRUE
PCSK1N	30	1	-0.5472	DOWN	TRUE
HRH1	31	1	-0.4651	DOWN	TRUE
MAFB	32	1	-0.495	DOWN	TRUE
KRT15	33	1	-0.4449	DOWN	TRUE
SERPINI1	34	1	-0.4156	DOWN	TRUE
ANXA1	35	1	0.4655	UP	FALSE
PTBP2	36	1	-0.4541	DOWN	TRUE
KCNF1	37	1	-0.4506	DOWN	TRUE

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STMN2	38	1	-0.4935	DOWN	TRUE
ISL1	39	1	-0.413	DOWN	TRUE
S100P	40	1	0.3866	UP	FALSE
SALL1	41	1	0.3582	UP	FALSE
CALB2	42	1	-0.4103	DOWN	TRUE
ST18	43	1	-0.3563	DOWN	TRUE
CXCL13	44	1	-0.3761	DOWN	FALSE
PDZK1	45	1	-0.3045	DOWN	TRUE
CADPS	46	1	-0.5	DOWN	TRUE
NEUROD2	47	1	-0.3869	DOWN	TRUE
NBEA	48	1	-0.3955	DOWN	TRUE
RIMBP2	49	1	-0.3592	DOWN	TRUE
CYP2C8	50	1	0.308	UP	FALSE
RASGRP1	51	1	-0.4649	DOWN	TRUE
ALDOB	52	1	0.5217	UP	TRUE
ALB	53	1	0.6417	UP	TRUE
FAM155B	54	1	-0.4511	DOWN	TRUE
DCX	55	1	-0.2911	DOWN	TRUE
ERO1LB	56	1	-0.2669	DOWN	TRUE
MMP1	57	1	0.4393	UP	FALSE
CDKN1C	58	1	-0.5125	DOWN	TRUE
KLK12	59	1	-0.3883	DOWN	TRUE
S100A1	60	1	-0.436	DOWN	TRUE
SCG5	61	1	-0.4433	DOWN	TRUE
PNLIP	62	1	0.7961	UP	TRUE
KCTD12	63	1	-0.4247	DOWN	TRUE
ZNF238	64	1	-0.3071	DOWN	TRUE
EMP2	65	1	-0.3854	DOWN	TRUE
CUZD1	66	1	0.7376	UP	TRUE
NID1	67	1	-0.4153	DOWN	TRUE
CFH	68	1	0.3814	UP	FALSE
PLAC8	69	1	-0.3035	DOWN	TRUE
MATN2	70	1	-0.2901	DOWN	TRUE
AADAC	71	1	0.4423	UP	TRUE
PAX4	72	1	-0.342	DOWN	TRUE
LAPTM5	73	1	-0.3558	DOWN	TRUE
HSPA2	74	1	-0.4773	DOWN	TRUE
SLC18A1	75	1	-0.3023	DOWN	TRUE
TSPAN7	76	1	-0.3948	DOWN	TRUE
KIAA1324	77	1	-0.247	DOWN	TRUE
CEL	78	1	0.6615	UP	TRUE
ELMO1	79	1	-0.3486	DOWN	TRUE
OLFML2B	80	1	-0.2589	DOWN	TRUE
ANPEP	81	1	0.4711	UP	FALSE
APOBEC2	82	1	-0.3499	DOWN	FALSE
PMP22	83	1	-0.265	DOWN	FALSE
ATHL1	84	1	0.3286	UP	FALSE

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SST	85	1	-0.5348	DOWN	FALSE
C14orf132	86	1	-0.413	DOWN	TRUE
FGL1	87	1	0.502	UP	TRUE
CPE	88	1	-0.5021	DOWN	TRUE
CPA1	89	1	0.6296	UP	TRUE
BTBD3	90	1	-0.3829	DOWN	TRUE
MLLT11	91	0.99999	-0.4209	DOWN	TRUE
DNAJC12	92	0.99999	-0.3083	DOWN	FALSE
8-Mar	93	0.99999	-0.3467	DOWN	TRUE
FKBP11	94	0.99998	0.4237	UP	TRUE
MUC1	95	0.99998	0.3444	UP	TRUE
CALY	96	0.99997	-0.2487	DOWN	TRUE
LGALS2	97	0.99997	0.4254	UP	TRUE
PDIA2	98	0.99996	0.4374	UP	TRUE
ATP2A3	99	0.99996	-0.3128	DOWN	TRUE
HHLA2	100	0.99996	0.2253	UP	TRUE
CPA2	101	0.99996	0.6583	UP	TRUE
CLGN	102	0.99996	-0.2043	DOWN	TRUE
DPYSL4	103	0.99995	-0.237	DOWN	TRUE
EYA2	104	0.99995	-0.2663	DOWN	TRUE
ANXA10	105	0.99994	0.3769	UP	TRUE
RRAD	106	0.99994	-0.2087	DOWN	FALSE
PTK6	107	0.99991	0.2373	UP	TRUE
PRSS3	108	0.99988	0.5719	UP	TRUE
G0S2	109	0.99984	0.3344	UP	FALSE
PTX3	110	0.99979	-0.1942	DOWN	TRUE
CLPS	111	0.99979	0.628	UP	FALSE
KIT	112	0.99978	-0.2826	DOWN	TRUE
QPCT	113	0.99964	-0.3921	DOWN	TRUE
MXRA5	114	0.99962	0.3479	UP	TRUE
TBPL1	115	0.99958	-0.322	DOWN	TRUE
C7	116	0.99953	-0.2227	DOWN	TRUE
INA	117	0.99915	-0.3138	DOWN	TRUE
DPP6	118	0.99897	-0.255	DOWN	TRUE
REG3A	119	0.99893	0.6308	UP	TRUE
PCMTD2	120	0.99867	-0.2984	DOWN	FALSE
APLP1	121	0.99858	-0.316	DOWN	TRUE
HMGB3	122	0.99854	-0.3289	DOWN	TRUE
HIST1H1C	123	0.9985	0.299	UP	TRUE
LDHB	124	0.99849	0.4463	UP	TRUE
NQO1	125	0.99847	0.2713	UP	TRUE
PLA2G1B	126	0.99823	0.5601	UP	TRUE
RAP1GAP2	127	0.99811	-0.2499	DOWN	TRUE
NPC1L1	128	0.99771	-0.2481	DOWN	TRUE
SYT11	129	0.99758	-0.2571	DOWN	TRUE
KLHL3	130	0.99733	-0.2507	DOWN	TRUE
GDAP1	131	0.99705	-0.2096	DOWN	FALSE

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SLC17A7	132	0.99698	-0.2725	DOWN	TRUE
DDC	133	0.99657	-0.2336	DOWN	TRUE
FEV	134	0.99651	-0.3317	DOWN	TRUE
MUC13	135	0.99649	0.3344	UP	FALSE
AC015936.3	136	0.99641	0.2092	UP	FALSE
XRCC4	137	0.99622	0.1936	UP	TRUE
FAM105A	138	0.99604	-0.212	DOWN	TRUE
ALAS2	139	0.996	-0.2332	DOWN	TRUE
RBP4	140	0.99502	-0.3002	DOWN	TRUE
KLK1	141	0.99476	0.4035	UP	TRUE
PTPRE	142	0.99401	-0.1767	DOWN	FALSE
SSTR2	143	0.99371	-0.2322	DOWN	TRUE
AE000659.9	144	0.99324	-0.1899	DOWN	FALSE
AGPAT5	145	0.99272	0.2752	UP	TRUE
RNASE1	146	0.99269	0.3817	UP	TRUE
NOL4	147	0.99232	-0.194	DOWN	TRUE
A1CF	148	0.99169	-0.2166	DOWN	FALSE
LDLRAP1	149	0.99168	-0.2814	DOWN	TRUE
NPTXR	150	0.99113	-0.228	DOWN	TRUE
SSU_rRNA_5	151	0.99063	0.262	UP	FALSE
MYC	152	0.99042	0.2907	UP	TRUE
PNLIPRP1	153	0.99036	0.3672	UP	TRUE
ZNF165	154	0.99036	0.2497	UP	TRUE
DIRAS3	155	0.99006	-0.214	DOWN	FALSE
PCYOX1L	156	0.98907	-0.2549	DOWN	TRUE
SFN	157	0.98893	0.3179	UP	TRUE
AMOTL2	158	0.98831	-0.2712	DOWN	TRUE
TSPYL5	159	0.98826	-0.2697	DOWN	TRUE
ARHGAP22	160	0.98318	-0.2327	DOWN	TRUE
CACNA1B	161	0.98205	-0.2034	DOWN	TRUE
TMEM35	162	0.98183	-0.1669	DOWN	TRUE
RGS2	163	0.98001	0.2665	UP	FALSE
ORM1	164	0.97882	0.2285	UP	FALSE
RFTN1	165	0.97853	-0.2199	DOWN	FALSE
SSTR1	166	0.97714	-0.1961	DOWN	TRUE
CNGA3	167	0.97666	-0.1664	DOWN	TRUE
SH3GL2	168	0.97454	-0.1742	DOWN	TRUE
C4BPB	169	0.97378	0.2128	UP	TRUE
ANGPT2	170	0.97356	0.157	UP	TRUE
DMXL2	171	0.97144	-0.1825	DOWN	TRUE
CBFA2T2	172	0.96808	-0.2341	DOWN	TRUE
REG1P	173	0.96115	0.2766	UP	TRUE
F3	174	0.96102	0.303	UP	TRUE
SETBP1	175	0.96073	-0.2286	DOWN	TRUE
FAM129A	176	0.95967	0.2818	UP	TRUE
HCP5	177	0.95864	-0.2421	DOWN	TRUE

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GNS	178	0.95786	-0.2821	DOWN	TRUE
HIST1H2AK	179	0.95628	0.1615	UP	TRUE
PLCG2	180	0.95602	-0.225	DOWN	TRUE
AKR1C3	181	0.95122	0.3164	UP	FALSE
CPA4	182	0.9504	0.2841	UP	FALSE
AGR2	183	0.94784	0.2142	UP	TRUE
RP3-522P13.3	184	0.94545	0.1743	UP	TRUE
CDA	185	0.94299	0.1972	UP	FALSE
RGS7	186	0.94005	-0.1425	DOWN	TRUE
HSD11B2	187	0.93722	-0.2117	DOWN	FALSE
NHLH2	188	0.93696	-0.1789	DOWN	TRUE
GPRASP1	189	0.93652	-0.1664	DOWN	TRUE
SPRR1A	190	0.93651	0.2303	UP	TRUE
CP	191	0.93335	0.1729	UP	FALSE
PRSS1	192	0.9258	0.535	UP	TRUE
BACE1	193	0.92461	-0.2707	DOWN	TRUE
TIMP3	194	0.9207	-0.2325	DOWN	FALSE
CCNA1	195	0.91809	-0.1743	DOWN	TRUE
DPYSL3	196	0.91799	-0.2349	DOWN	TRUE
MOCOS	197	0.90975	0.1968	UP	TRUE
CDK5R2	198	0.90515	-0.1574	DOWN	TRUE
SIM1	199	0.90446	-0.2259	DOWN	TRUE
KYNU	200	0.88769	0.1434	UP	TRUE
CASD1	201	0.88049	-0.1978	DOWN	TRUE
HIST1H2BK	202	0.86053	0.2884	UP	TRUE
GIF	203	0.85397	-0.1795	DOWN	TRUE
MAP2K6	204	0.84399	-0.181	DOWN	TRUE
DOHH	205	0.84281	0.1952	UP	TRUE
ARL14	206	0.84037	0.1569	UP	TRUE
SCT	207	0.83551	0.1439	UP	FALSE
HIST1H4B	208	0.81992	0.1253	UP	FALSE
UPK1B	209	0.81779	0.2332	UP	TRUE
ZBTB10	210	0.81437	-0.1205	DOWN	TRUE
EFHD2	211	0.81025	-0.2075	DOWN	TRUE
RHOBTB3	212	0.80403	-0.2389	DOWN	TRUE
MALL	213	0.80066	0.3275	UP	TRUE
PRAMEF11	214	0.79927	0.1376	UP	TRUE
GABRE	215	0.79827	0.1628	UP	TRUE
COL16A1	216	0.79786	-0.2066	DOWN	TRUE
WDR47	217	0.79434	-0.1804	DOWN	FALSE
AOX1	218	0.79226	0.261	UP	TRUE
PLTP	219	0.78478	0.2448	UP	TRUE
ANKRD46	220	0.77664	-0.191	DOWN	TRUE
CEACAM6	221	0.77573	0.2309	UP	FALSE
SACM1L	222	0.77211	-0.2071	DOWN	TRUE
AC015936.4	223	0.76722	-0.2501	DOWN	TRUE

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SLMO2	224	0.76655	-0.1281	DOWN	FALSE
RUNDC3A	225	0.76653	-0.1996	DOWN	TRUE
IL1A	226	0.75949	0.1756	UP	FALSE
ARHGEF9	227	0.75449	-0.1797	DOWN	TRUE
ECEL1	228	0.75425	-0.2269	DOWN	TRUE
FBXO2	229	0.7535	0.2227	UP	TRUE
TCF15	230	0.74955	0.1775	UP	TRUE
IGF2	231	0.74558	0.2166	UP	TRUE
FOSL1	232	0.74293	0.1831	UP	FALSE
RGS4	233	0.72933	-0.3263	DOWN	FALSE
TCN1	234	0.72866	0.3125	UP	FALSE
KAT2B	235	0.72781	-0.1668	DOWN	TRUE
ST3GAL5	236	0.72649	-0.203	DOWN	TRUE
RCBTB1	237	0.71116	-0.195	DOWN	FALSE
PEG10	238	0.70577	-0.2105	DOWN	TRUE
INHBE	239	0.70454	-0.1027	DOWN	TRUE
ENOX1	240	0.7012	-0.1491	DOWN	TRUE
AC115097.1	241	0.70099	0.1271	UP	FALSE
THSD7A	242	0.6969	0.1411	UP	TRUE
C6orf211	243	0.6947	-0.1414	DOWN	TRUE
LTF	244	0.69008	-0.1688	DOWN	FALSE
ZNF787	245	0.68968	0.1786	UP	TRUE
THOP1	246	0.66221	0.1738	UP	TRUE
HIST1H2BI	247	0.66171	0.1719	UP	TRUE
AKR1C2	248	0.65151	0.2206	UP	FALSE
APBA2	249	0.6391	-0.1886	DOWN	TRUE
AMDHD2	250	0.63635	0.158	UP	TRUE
DLK1	251	0.63078	-0.319	DOWN	FALSE
PRSS21	252	0.62707	0.1587	UP	FALSE
CD68	253	0.6268	0.1206	UP	FALSE
NEB	254	0.62144	0.1583	UP	TRUE
NARG2	255	0.61847	-0.1513	DOWN	FALSE
EIF2AK3	256	0.61668	-0.1229	DOWN	FALSE
TAF9B	257	0.61266	-0.1483	DOWN	TRUE
CHD7	258	0.60554	-0.1817	DOWN	FALSE
CXCL3	259	0.5978	0.1842	UP	TRUE
PAPSS2	260	0.5972	-0.1831	DOWN	TRUE
RANBP2	261	0.58572	-0.1795	DOWN	TRUE
AL137849.3	262	0.58404	0.1409	UP	TRUE
TBC1D30	263	0.58094	-0.1274	DOWN	TRUE
CCL7	264	0.58014	0.132	UP	TRUE
TGFBR1	265	0.57586	-0.1669	DOWN	TRUE
GSTA1	266	0.57576	0.3761	UP	TRUE
CRCT1	267	0.56948	0.1714	UP	FALSE
PGAM2	268	0.56936	-0.1611	DOWN	TRUE
KIR2DL1	269	0.56919	0.1281	UP	FALSE
HIST1H2BE	270	0.56379	0.1446	UP	TRUE

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TFF2	271	0.56286	0.1785	UP	TRUE
HOPX	272	0.56189	-0.1525	DOWN	TRUE
TBC1D4	273	0.55783	-0.2262	DOWN	TRUE
CDKN2C	274	0.55511	-0.2054	DOWN	TRUE
C5orf44	275	0.55275	-0.1232	DOWN	TRUE
FUT4	276	0.54634	0.2463	UP	TRUE
PDGFRL	277	0.54212	0.1787	UP	TRUE
AC093698.2	278	0.53514	0.2097	UP	FALSE
KANK1	279	0.53433	-0.2331	DOWN	TRUE
C10orf116	280	0.53357	0.1613	UP	TRUE
SCN1B	281	0.52567	-0.1972	DOWN	TRUE
MYH7	282	0.51515	0.1496	UP	FALSE
TAGLN3	283	0.50947	-0.1942	DOWN	TRUE
TMEM97	284	0.50635	-0.2136	DOWN	TRUE
TYRP1	285	0.4984	0.1436	UP	FALSE
PUS1	286	0.48846	0.1509	UP	TRUE
ABCC8	287	0.48487	-0.1556	DOWN	TRUE
NFASC	288	0.48075	-0.1604	DOWN	TRUE
ASRGL1	289	0.45283	-0.2088	DOWN	TRUE
AC010265.1	290	0.44527	0.0993	UP	FALSE
FCGR2A	291	0.44373	0.124	UP	FALSE
STMN4	292	0.44267	-0.1797	DOWN	TRUE
TOX	293	0.43356	-0.122	DOWN	TRUE
INPP4B	294	0.42844	-0.1303	DOWN	FALSE
TPST2	295	0.42622	0.2766	UP	TRUE
DUSP6	296	0.4258	0.1872	UP	TRUE
PCDH1	297	0.41533	0.1635	UP	TRUE
CEP170	298	0.41515	-0.1866	DOWN	TRUE
F5	299	0.41366	0.172	UP	TRUE
C3orf14	300	0.41307	-0.2362	DOWN	TRUE
SV2A	301	0.40515	-0.1937	DOWN	TRUE
RPAP3	302	0.40273	-0.1283	DOWN	TRUE
CTSE	303	0.39365	0.2521	UP	TRUE
ZZZ3	304	0.38283	-0.2037	DOWN	TRUE
PGM3	305	0.38126	0.1805	UP	TRUE
HTR3A	306	0.37765	0.17	UP	TRUE
CTSC	307	0.37557	0.2289	UP	FALSE
SERPINI2	308	0.36883	0.3342	UP	TRUE
ALDOC	309	0.36302	-0.2266	DOWN	TRUE
HIST2H2BE	310	0.35641	-0.2008	DOWN	TRUE
CSF1R	311	0.35623	-0.1575	DOWN	FALSE
PIK3C2A	312	0.32286	-0.1774	DOWN	TRUE
BTN2A2	313	0.31997	-0.1448	DOWN	TRUE
CHRNA5	314	0.31974	-0.1322	DOWN	FALSE
PLK2	315	0.30546	0.1876	UP	TRUE
SLPI	316	0.30342	0.2896	UP	TRUE
GULP1	317	0.30308	0.2174	UP	TRUE

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NEUROG1	318	0.29195	-0.1438	DOWN	TRUE
MEX3C	319	0.27804	-0.1351	DOWN	FALSE
BOLA1	320	0.27356	0.1255	UP	TRUE
HSD17B2	321	0.26978	0.2359	UP	TRUE
MID1IP1	322	0.26438	-0.232	DOWN	TRUE
STXBP2	323	0.26333	0.1959	UP	TRUE
ITGB6	324	0.2614	-0.1805	DOWN	TRUE
GNMT	325	0.26057	0.1857	UP	FALSE
EMP1	326	0.25626	0.1557	UP	TRUE
PVRL3	327	0.25486	-0.1337	DOWN	TRUE
ISOC1	328	0.25428	-0.1674	DOWN	TRUE
ZNF205	329	0.25352	0.1474	UP	TRUE
MKRNP5	330	0.2519	-0.1245	DOWN	FALSE
PWP2H	331	0.25008	0.157	UP	TRUE
CORO2B	332	0.24683	-0.2133	DOWN	FALSE
GLS	333	0.24673	-0.1362	DOWN	TRUE
GADD45G	334	0.24643	-0.1291	DOWN	FALSE
C20orf91	335	0.24328	0.1388	UP	TRUE
PITPNM1	336	0.23742	0.1673	UP	TRUE
PCSK2	337	0.23707	-0.1372	DOWN	TRUE
RSBN1	338	0.23277	-0.138	DOWN	TRUE
ELAVL4	339	0.23265	-0.1486	DOWN	TRUE
TNFSF12	340	0.23218	0.1676	UP	TRUE
FZD6	341	0.22997	-0.1798	DOWN	TRUE
CRYAB	342	0.22889	0.252	UP	FALSE
CDKAL1	343	0.22735	-0.1399	DOWN	FALSE
ACAD8	344	0.22427	-0.1709	DOWN	TRUE
PRKD3	345	0.21976	-0.1527	DOWN	TRUE
ZNHIT6	346	0.21464	-0.1304	DOWN	TRUE
CLDN5	347	0.21258	0.1176	UP	TRUE
ITGA3	348	0.21176	0.1991	UP	TRUE
LGALS4	349	0.21144	0.2851	UP	TRUE
SNAI2	350	0.20991	-0.1156	DOWN	TRUE
MYCL1	351	0.2081	-0.1503	DOWN	TRUE
FAM46A	352	0.20771	0.218	UP	TRUE
SUZ12	353	0.20659	-0.1906	DOWN	TRUE
EFNB2	354	0.2035	0.1665	UP	TRUE
AKR1B10	355	0.20196	0.2452	UP	TRUE
CDK2AP2	356	0.20116	0.1472	UP	TRUE
NMNAT2	357	0.19792	-0.1609	DOWN	TRUE
SLC35A5	358	0.19743	-0.1626	DOWN	FALSE
FKTN	359	0.19699	-0.1353	DOWN	FALSE
PRLH	360	0.19627	0.1352	UP	TRUE
CHUK	361	0.19593	-0.1317	DOWN	TRUE
SHARPIN	362	0.19171	0.1676	UP	TRUE
CTA-246H3.3	363	0.19131	0.1275	UP	FALSE

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SOX2	364	0.19061	-0.1467	DOWN	TRUE
TM7SF2	365	0.18796	0.2059	UP	TRUE
ISG20	366	0.18688	0.1894	UP	TRUE
IQCC	367	0.18514	0.1392	UP	TRUE
CSRP3	368	0.18309	0.111	UP	FALSE
STEAP3	369	0.18162	-0.1976	DOWN	TRUE
GCNT3	370	0.17977	0.2102	UP	TRUE
FZD2	371	0.17944	-0.1162	DOWN	FALSE
GLT25D2	372	0.17813	-0.1821	DOWN	FALSE
RALGAPA1	373	0.17557	-0.1495	DOWN	TRUE
ZNF43	374	0.17548	-0.1058	DOWN	TRUE
PLAT	375	0.1748	0.1178	UP	FALSE
STON1	376	0.17248	-0.1137	DOWN	TRUE
ARNT2	377	0.17219	-0.1964	DOWN	TRUE
NGF	378	0.1708	0.1298	UP	TRUE
FAM174B	379	0.16894	-0.1415	DOWN	TRUE
GIPC2	380	0.16877	-0.1199	DOWN	TRUE
NRIP1	381	0.1685	-0.1509	DOWN	TRUE
SNAP25	382	0.16243	-0.2478	DOWN	TRUE
BAZ2B	383	0.15956	-0.1881	DOWN	TRUE
GCA	384	0.15952	-0.1696	DOWN	TRUE
RASL11B	385	0.15865	-0.1363	DOWN	TRUE
WASF3	386	0.15692	-0.1449	DOWN	FALSE
IGLV4-60	387	0.15517	0.1255	UP	FALSE
LIN7C	388	0.15225	-0.1499	DOWN	TRUE
GPRC5A	389	0.15194	0.1784	UP	TRUE
PTMAP1	390	0.15174	-0.1067	DOWN	FALSE
BEGAIN	391	0.14717	0.15	UP	FALSE
PREPL	392	0.14549	-0.2003	DOWN	TRUE
TFF1	393	0.14472	0.1429	UP	TRUE
ADAMTS5	394	0.14374	-0.1132	DOWN	TRUE
FGB	395	0.14318	0.1728	UP	TRUE
SLC22A3	396	0.142	-0.1048	DOWN	TRUE
HERC1	397	0.14172	-0.1617	DOWN	TRUE
CSNK1G3	398	0.14077	-0.1608	DOWN	TRUE
PTPRN2	399	0.14003	-0.1707	DOWN	TRUE
NFKBIL2	400	0.13849	0.1081	UP	TRUE

Supplementary table:2. Differentially significant genes of both classes of case and control studies with $p<0.05$

BothClasses.GeneName	BothClasses.ranking	BothClasses.postProb	BothClasses.exprsMeanDiff	BothClasses.exprsUpDw	BothClasses.isRedundant
HEPACAM2	1	1	-1.4592	DOWN	FALSE
HES6	2	1	-0.7198	DOWN	TRUE
CPLX1	3	1	-0.728	DOWN	FALSE
SSTR1	4	1	-0.7028	DOWN	TRUE
TMED6	5	1	-0.5992	DOWN	FALSE

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SUSD2	6	1	-0.6146	DOWN	TRUE
CACNA2D1	7	1	-0.6781	DOWN	TRUE
KRTAP9-4	8	1	-0.5061	DOWN	TRUE
CACNA1B	9	1	-0.4666	DOWN	TRUE
KRTAP3-3	10	1	-0.4678	DOWN	TRUE
SALL1	11	1	0.2984	UP	FALSE
ERO1LB	12	1	-0.4529	DOWN	TRUE
KCNK17	13	1	-0.5178	DOWN	TRUE
PAX6	14	1	-0.3063	DOWN	TRUE
DACH1	15	1	-0.3532	DOWN	TRUE
C19orf30	16	1	-0.3425	DOWN	TRUE
TCERG1L	17	1	0.3168	UP	FALSE
TMEM61	18	1	-0.4383	DOWN	TRUE
EFCAB7	19	1	-0.3193	DOWN	FALSE
NOL4	20	1	-0.2485	DOWN	FALSE
NAP1L5	21	0.99999	-0.3035	DOWN	TRUE
GDAP1	22	0.99998	-0.3248	DOWN	TRUE
LFNG	23	0.99995	-0.3709	DOWN	TRUE
NANOS1	24	0.99991	-0.2233	DOWN	TRUE
MCC	25	0.9995	-0.2176	DOWN	TRUE
SSU_rRNA_5	26	0.9995	0.447	UP	FALSE
SOX2	27	0.99943	-0.2473	DOWN	TRUE
GLS	28	0.99931	-0.2741	DOWN	TRUE
AC140481.3	29	0.99915	-0.2842	DOWN	TRUE
SNORND104	30	0.99829	-0.2478	DOWN	FALSE
SLC25A34	31	0.99624	-0.2758	DOWN	TRUE
SYT4	32	0.9932	-0.2488	DOWN	TRUE
MANEA	33	0.99234	-0.1887	DOWN	TRUE
KRTAP4-1	34	0.99016	-0.1891	DOWN	FALSE
MGST1	35	0.98971	0.4894	UP	FALSE
PLCXD3	36	0.98624	-0.2594	DOWN	TRUE
GPR123	37	0.98589	-0.2262	DOWN	TRUE
AC108047.1	38	0.98548	0.2356	UP	FALSE
AL035610.1	39	0.98085	0.2416	UP	TRUE
HMGB3	40	0.97723	-0.3161	DOWN	TRUE
AL035460.2	41	0.97517	0.1442	UP	FALSE
NBEA	42	0.97465	-0.2992	DOWN	TRUE
AC087749.3	43	0.97309	-0.3324	DOWN	TRUE
NOSTRIN	44	0.969	0.2645	UP	TRUE
MOBKL1A	45	0.95906	-0.2925	DOWN	TRUE
RP3-475B7.1	46	0.95778	0.2069	UP	FALSE

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Supplementary Table: 3

Description	P-value	FDR q-value	Enrichment	Genes
response to monosaccharide	2.46E-05	1.04E-01	57.21	NEUROD1, NPTX1, NKX2-2
endocrine pancreas development	2.46E-05	5.19E-02	57.21	NEUROD1, NKX2-2,
response to hexose	2.46E-05	3.46E-02	57.21	NEUROD1, NKX2-2, NPTX1
response to glucose	2.46E-05	2.60E-02	57.21	NEUROD1, NPTX1, NKX2-2
regulation of neural precursor cell proliferation	3.63E-05	3.07E-02	11.12	SALL1, INSM1, PAX6, PTBP2, ASCL1,
response to carbohydrate	3.75E-05	2.64E-02	50.06	NEUROD1, NPTX1, NKX2-2
cell fate determination	3.75E-05	2.27E-02	14.05	NKX2-2, PAX6, ISL1, ASCL1
type B pancreatic cell development	4.22E-05	2.23E-02	178	NKX2-2, INSM1
glandular epithelial cell differentiation	5.09E-05	2.39E-02	16.43	NEUROD1, INSM1, PAX6, ASCL1
pancreatic A cell fate commitment	8.43E-05	3.56E-02	133.5	NEUROD1, NKX2-2
pancreatic PP cell fate commitment	8.43E-05	3.24E-02	133.5	NEUROD1, NKX2-2
epithelial cell fate commitment	8.43E-05	2.97E-02	133.5	NEUROD1, NKX2-2
positive regulation of neuron differentiation	1.12E-04	3.63E-02	7.74	SALL1, NEUROD1, NEUROG3, NEUROD2, NKX2-2, ASCL1
columnar/cuboidal epithelial cell development	1.22E-04	3.68E-02	118.67	NKX2-2, INSM1
glandular epithelial cell development	1.22E-04	3.43E-02	118.67	NKX2-2, INSM1
cellular response to glucose stimulus	1.31E-04	3.46E-02	22.25	NEUROD1, NPTX1, UCP2
cellular response to hexose stimulus	1.31E-04	3.26E-02	22.25	NEUROD1, NPTX1, UCP2 -
cellular response to monosaccharide stimulus	1.31E-04	3.07E-02	22.25	NEUROD1, NPTX1, UCP2
glial cell fate specification	1.63E-04	3.64E-02	20.54	NKX2-2, PAX6, ASCL1
oligodendrocyte cell fate specification	1.63E-04	3.45E-02	20.54	NKX2-2, PAX6, ASCL1
enteroendocrine cell	1.63E-04	3.29E-02	20.54	NEUROD1, INSM1,

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differentiation				PAX6
regulation of neuron differentiation	1.72E-04	3.31E-02	4.98	SALL1, NEUROG1, NEUROD1, NEUROD2, NKX2-2, PAX6, ISL1, ASCL1, STMN2
epithelial cell development	3.33E-04	6.13E-02	89	NKX2-2, INSM1
neurogenesis	4.15E-04	7.30E-02	15.71	NEUROD1, ANXA1, ASCL1
carbohydrate homeostasis	5.68E-04	9.60E-02	11.74	NEUROD1, NPTX1, PAX6, UCP2
glucose homeostasis	5.68E-04	9.23E-02	11.74	NEUROD1, NPTX1, PAX6, UCP2
cellular response to carbohydrate stimulus	5.74E-04	8.98E-02	66.75	NEUROD1, NPTX1
regulation of neurogenesis	6.86E-04	1.04E-01	4.35	SALL1, NEUROG3, NEUROD1, NEUROD2, NKX2-2, PAX6, ISL1, ASCL1, STMN2,
positive regulation of neural precursor cell proliferation	9.27E-04	1.35E-01	15.4	INSM1, PAX6, ASCL1

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3D STRUCTURE PREDICTION AND IDENTIFICATION OF SMART DOMAIN SEQUENCES OF THYMIDINE KINASE OF CAPRIPOX VIRUS: AN *INSILICO* APPROACH.

[1]KiranKumar Burranboina,*[1,3]Kumar K M, [2]Manjunatha Reddy G B, [2]Yogisharadhya R, [3]Gomathi R, [3]Rupali Sinha, [3]Deepti Kiran.

[1]Department of Biotechnology, REVA University, Bengaluru, India.
[2]National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India
[3]Dayananda Sagar college of engineering, Bengaluru, India.

ABSTRACT: The sheeppox virus (SPPV) and goatpox virus (GTPV) and lymphy skin disease viruses are contagious viral skin diseases of sheep, goat and cattles. They are members of the Poxviridae, genus Capripoxvirus These viruses are belived that same strains of the same virus, but genetic sequencing has shown they are closely related separate viruses. Theses are the economically important diseases which collectively constitute the most serious poxvirus diseases of production animals. vaccine strains of the capripoxvirus and their specice specific importance these viruses were used in new vaccine vector development. Thymidine kinase is the enzyme that catalyzes the ATP-dependent phosphorylation of thymidine. The viral thymidine kinase (TK) gene as the site of foreign gene insertion as well used to construct recombinat vector to develop effective vaccines for various diseases veterinary importance. The results of this study Structure Prediction, identification of SMART domain sequences of thymidine kinase and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future recombinant vaccine, vector and drug designing.

Keywords: Thymidine kinase, Capripox virus and SMART (Simple Modular Architecture Research Tool).

***Corresponding Author:** KiranKumar Burranboina, Research scholar, REVA University, Bangalore. Mail id: kirankumarq@gmail.com

1. INTRODUCTION

Sheeppox virus, Goatpox virus and Lymphyskin disease virus are member of genus Capripoxvirus in the family Poxviridae, causes sheeppox, Goatpox and Lymphyskin disease in Sheep, Goat and Cattle respectively. These are contagious viral disease of domestic animals and ancient diseases that are currently endemic in the Middle East, southwest and central Asiaand the Indian subcontinent and Northern Africa. Kids and lambs are generally more susceptible than adults [1],[2] and 3]. Sheeppox, goatpox and Lymphy skin diseases are the viral diseases exhibit similar clinical signs like a malignant systemic disease with high temperature, ocular and oronasal discharges, typical of generalized poxviral diseases, cutaneous lesions, including pyrexia, and notably the development of lung lesions [3], [4]. The capripoxviruses isolated from sheep and goats in Kenya are not host specific as in the Middle East and India. The same virus appears to occur in field outbreaks in mixed flocks and shown one species are of a similar pathogenicity [5]. The disease causes noteworthy economic losses by reduced hide and wool quality [6].

These diseases are listed diseases by the World Organization for Animal Health (OIE) [7] And these are the systemic transmissible viral diseases characterized mainly by skin and

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internal lesions [8]. Capripoxviral diseases are the Livestock diseases continue to pose a major threat to the world food supply as well as the agricultural industry at large[9], [10]. The case fatality 10 - 85% and morbidity is 75-100% aepending on the virulence of the virus[11]. Capripoxviruses are large (170–260 nm by 300-450 nm), enveloped and double-stranded DNA viruses with genomes approximately 150–160 kbp nucleotides long and has termini with cross-linked hairpins[12], [13], [14].

The aim of this work was capripox viruses are the highly contagious viral diseases. Thymidine kinase gene nonessential region used for foreign gene insertion for selecting recombinant viruses and the TK gene of vaccinia virus mostly extensively studied orthopoxvirus[23], By targeting the thymidine kinase (TK) gene structural Prediction and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

2. MATERIALS AND METHODS

2.1 Protein structure Sequence retrieval:

The *sheeppox* virus Thymidine kinase sequence was retrived from https://www.uniprot.org in FASTA format and used for further analyses. The accession number are >sp|P16600|KITH_SHEVK Thymidine kinase.

2.2 Physico-chemical and secondary structural characterization: The Physico-chemical characterization of Thymidine kinase of sheep pox was carried out by using Expasy's ProtParam server (http://us.expasy.org/tools/protparam.html). To calculate the secondary structural features of the protein sequence was carried out by using SOPMA tools [15],[16], [17]. And the results were tabulated in Table 1.

2.3 Homology modeling and structural validation: Homology modeling and 3D structural prediction of Thymidinekinase protein was carried out by using phyre2, (http://www.sbg.bio.ic.ac.uk/phyre2) [18, 19] which uses advanced remote homology detection methods to build 3D models. The evaluation of protein structural models was performed by using PROCHECK, Swiss-PdbViewer software and Finally chosen model was visualized by Rasmol tools[20] ,[21].

2.4 Smart domain analysis: SMART is a in-house tool (Simple Modular Architecture Research Tool), allows users to quickly identification and annotation of protein domains and the exploration of protein domain architectures[22].

3. RESULTS AND DISCUSSION

3.1 Retrieval of protein sequence: The target nucleotide FASTA sequence of the Thymidine kinase of sheeppox virus complete cds attained.

>sp|P16600|KITH_SHEVK Thymidine kinase OS=Sheeppox virus (strain KS-1)
MDYGYIHLIIGPMFSGKSTELIRIVKRYQIAQYKCCVVKYLKDIRYGNSVYTHDNNHV
SAISTTLLYDVVDKIMNFDIIGIDEGQFFKDIVSFSENMANMGKIIIIAALDSTFQRKEF
NDILKLIPLSEKVTKLNAVCMECYKDAAFSKRITKEKEIELIGGKEKYKSVCRKCYFL
E

3.2 Physico-chemical and secondary structure characterization of Thymidine Kinase: To attain physicochemical and secondary structural properties like quantitative values for the amount of alpha-helices, beta sheets and coils present within the amino acid stretch of the sheeppox viral protein thymidine kinase by using SOPMA tool and Expasy's ProtParam tools was used and the results attained were tabulated in Table 1& 2.

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Table 1. Parameters computed using Expasy's ProtParam tool.		**Table 2 Calculated secondary structure elements by SOPMA.**	
Property	**Values**	**Secondary structure elements**	
Number of amino acides	177	Alpha helix (Hh)	40.68%
Molecular weight	20446.96	310 helix (Gg)	0.00%
Theoretical pl	8.62	Pi helix (Ii)	0.00%
Total number of negatively charged residues(Asp+Glu)	22	Beta bridge (Bb)	0.00%
Total number of positively residues (Arg+Lys)	26	Extended strand (Ee)	17.51%
Extinction coefficient (0.820, assuming all pairs of Cys residues form cystines)	16765	Beta turn (Tt)	8.47%
Extinction coefficient* (0.802, assuming all Cys residues are reduced)	16390	Bend region (Ss)	0.00%
Instability index	16.18	Random coil (Cc)	33.33%
Aliphatic index	97.46	Ambiguous states (?)	0.00%
Grand average of hdropathicity	-0.066	Other states	0.00%

3.3 Smart domain analysis: The sheeppox thymidine kinase structural domain identification was performed using the SMART online tool (http://smart.embl-heidelberg.) the result were shown below the table (Table 4). The outlier homologues and homologues of known structure TK shown three scop domain was charactise of Structural Classification of Proteins **2J87|D** - Superfamily: Transferase activity, Blast: **AAA**_R0HCI1_9BRAS 78-211: The AAA+ superfamily of ATPases is found in all kingdoms of living organisms where they participate in diverse cellular processes including membrane fusion, proteolysis and DNA replication and SCOP:d1j9ya - Superfamily: (Trans) glycosidases.

Table: 3 Outlier homologues and homologues of known structure:

Name	**SCOP domain (Structural Classification of Proteins)**	**Start**	**End**	**e-value**
PDB:2J87|D	Transferase	1	174	6.00E-74
Blast:AAA_R0HCI1_9BRAS 78-211	The AAA+ superfamily of ATPases.	4	128	5.00E-22
SCOP:d1j9ya_	Superfamily: (Trans)glycosidases	37	108	0.1

3.4 Structure prediction and evaluation: The 3D models of the target protein were constructed using Phyre2 homology model. Phyre2 is a online server which is used to predict and analyze protein structure, function and mutations. In this study the FASTA sequence of Thymidine kinase protein was entered and the intensive mode was selected to attain 3D models.The modelled and validated protein checked by PROCHECK the results were shown image 1, 2, 3.

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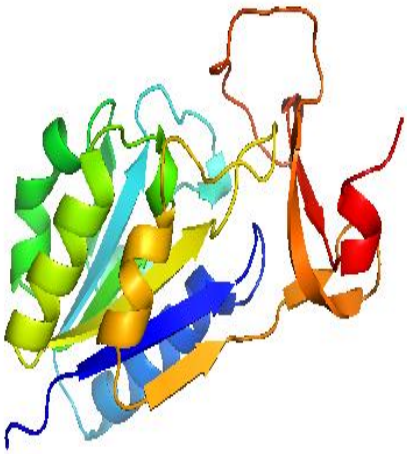


Figure. 1

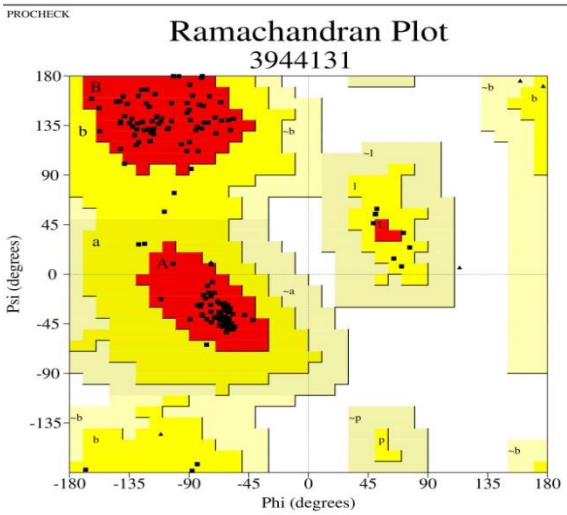
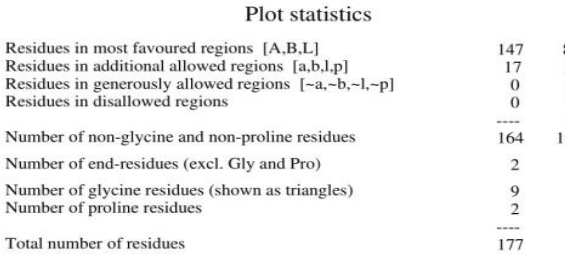


Figure. 2



Plot statistics

Residues in most favoured regions [A,B,L]	147	89.6%
Residues in additional allowed regions [a,b,l,p]	17	10.4%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	164	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	9	
Number of proline residues	2	
Total number of residues	177	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms or no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Figure. 3

4. CONCLUSION

In the present work, we predicted the 3D structures and SMART domain identification for thymidine kinase of sheeppox virus by various bioinformatics tools and servers. Knowing the structure, physicochemical properties and functional structural domains of protein is of great importance for understanding the molecular mechanisms of these proteins. As well this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

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A COMPARATIVE STUDY OF THE ANTI-FUNGAL ACTIVITY OF SOME SOUTH INDIAN MEDICINAL PLANTSAGAINST DIFFERENT FUNGAL PATHOGENS

Prathiba H.D *
Department of Biochemistry, Jnana Bharathi Campus, Bangalore University

ABSTRACT: The current work describes the antifungal activity of the five Indian medicinal plants against three human pathogenic fungi (Candida albicans, *Aspergillus niger* and *Trichoderma harzianum*). Antifungal assay was done using agar disc diffusion method.The result showed that the plant of five Indian medicinal plants i.e *Adathoda visca,Abutilon indicum*, *Datura stramonium*, *Lantana camara* and *Tridax procumbens*. The antifungal activity of the extracts increased linearly with increase in concentration of extracts.Amphotericin B (10µg/g) was used as standard drug for antifungal activity, Amphotericin B against *Aspergillus niger,* the zone of inhibition of 2.9 cm was noted, for *Candida albicans* 3.0 cm, for *Trichoderma harzianum* zone of inhibition of 3.5 cm was noted at an concentration of 10µg/mg. These extracts showed maximum activity, even at very low concentrations, and the same fungicide effects as chemical fungicide. We conclude from this that these extracts exhibit amazing fungicidal properties that support their traditional use as antiseptics.

KEYWORDS: Antifungal activity, Indian medicinal plants, fungal strains.

Corresponding Author: Dr. Prathiba H.D* Ph.D.
Department of Biochemistry, Jnana Bharathi Campus, Bangalore University.
Bangalore- 560056, India.
Email Address: prateeba8@gmail.com

1. INTRODUCTION

In developing countries and particularly in India low income people such as farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections [1]. Traditional healers claim that their medicine is cheaper and more effective than modem medicine. Patients of these communities have a reduced risk to get infectious diseases from resistant pathogens than people from urban are as treated with traditional antibiotics [2]. One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents. Traditional healers claim that some medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics.

Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide [3]. Human infections, particularly those involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries[4]. In humans, fungal infections range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases[5]. It is necessary to find out possible source for new potent antibiotics to which pathogen strains are not resistant. We chose five

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species used in folk medicine to determine their antifungal activity against clinical pathogens i.e. *Aspergillus niger* , *Candida albicans* and *Trichoderma harzianum*.

2. MATERIALS AND METHODS

2.1 Collection of plant material

On the basis of ethno botanical available literature and visual observation of plants that were relatively free from diseases and insect damages five plant species, *Adathoda visca,Abutilon indicum*, *Datura stramonium*, *Lantana camara* and *Tridax procumbens*. have been selected for the present study .The collected plant material were thoroughly washed and then dried under shade at 25± 2°C for about10 days. The dried plant samples were ground well into a fine powder in a mixer grinder. The powdered samples were then stored in air tight containers at room temperature.

2.2. Methanol extraction of Plant Material

Plant leaf powder (10 g) was placed in a 250 ml conical flask containing 100 ml of Methanol and plugged with cotton and placed on a rotary shaker (190-220 rpm for 24 h). Later, plant material-methanol mixture was filtered through 8 layers of muslin cloth and centrifuged at 5000 rpm for 15 min. The supernatant was collected and the solvent was evaporated to one-fourth of its original volume on water bath.

2.3. Preparation of inoculum

Stock cultures were maintained at 4°C on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 hrs at 37°C and 25°C respectively. The cultures were diluted with fresh Sabouraud dextrose broth to achieve optical densities corresponding to $2.0x10^6$ colony forming units (CFU/ml) for bacteria and 2.0×10^5 spores/ml for fungi.

2.4 Fungal Strains

The identified fungal strains were obtained from the National Chemical Laboratory (NCL), Pune, India. The fungal strains used in studies are *Aspergillus niger* NCIM 572, *Candida albicans* NCIM 3471 and *Trichoderma harzianum* NCIM 1185.

2.5Antifungal Activity

The antifungal assay was performed by disc diffusion method on potato dextrose agar culture medium [6]. The 20ml of sterilized potato dextrose agar medium was poured into sterile petriplates, after solidification, 100 µl of fresh fungal culture were swabbed on the respective plates. Each of discs approximately 5mm in diameter was cut from Whatman filter paper. The sterile discs were placed over the potato dextrose agar plates using sterile forceps and loaded with plant extracts at various concentrations (6, 8, and 10µg).The plates were incubated for 48 hours at 28°C. After incubation the diameter (cm) of inhibitory zones formed around each discs were measured.

3. RESULT AND DISCUSSION

Traditional medicines initially acquired the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations [7]. The specific plants to be used and the methods of application for particular ailments have been passed on through folk tradition. Plants with possible antifungal activity should be tested against some microbes to confirm the activity. The activity of plant extracts on fungi has been studied by a very large number of researchers in different parts of the world [8]. The selection of crude plant extracts for screening programs has the potential of being more successful in initial steps than the

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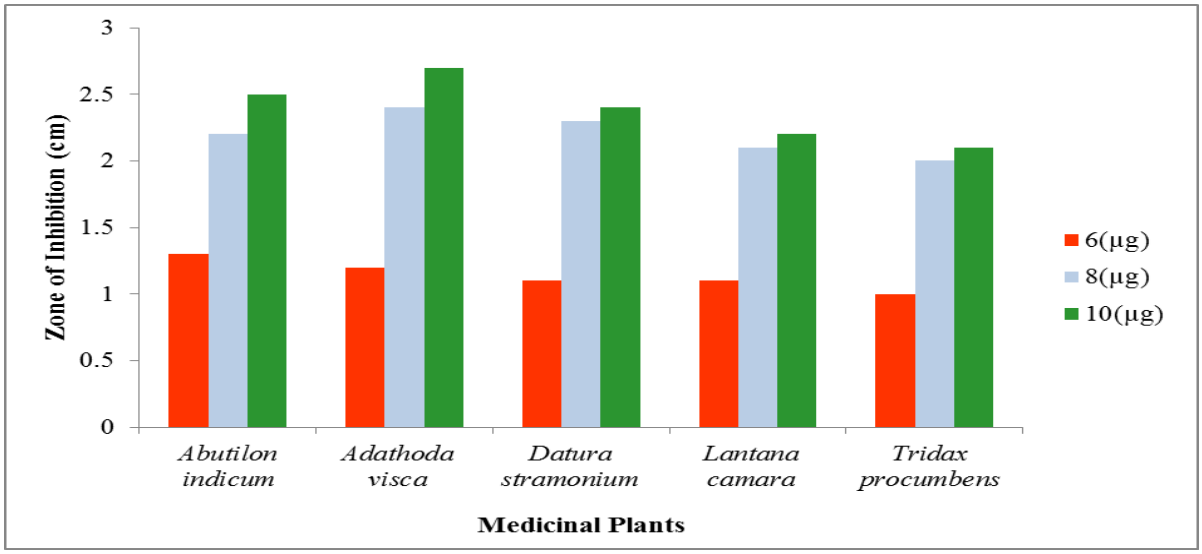
screening of pure compounds isolated from natural products [9]. The antifungal therapy is playing a greater role in health care and the screening of traditional plants for novel antifungals is now frequently performed. Fungal diseases represent a critical problem to health and they are one of the main causes of morbidity and mortality worldwide [10]. Human infections, particularly those `involving the skin and mucosal surfaces, constitute a serious problem, especially in tropical and subtropical developing countries [11]. In humans, fungal infections range from superficial to deeply invasive or disseminated, and have increased dramatically in recent years. The treatment of mycoses has lagged behind bacterial chemotherapy and fewer antifungal than antibacterial substances are available. Therefore, a search for new antifungal drugs is extremely necessary [12].

In the present study, the antifungal activities of methanol, extracts of five medicinal plants were investigated against dermatophytes and opportunistic pathogens.

3.1 Antifungal susceptibility test for alkaloid fraction from five medicinal plants.

Result obtained from the present study suggested that the antifungal activity of the alkaloids fraction of five different plants was determined against *Candida albicans*, (Figure 1). The zone of inhibition was highest as 1.2 cm at 6 µg/g, 2.4 cm at 8 µg/g, and 2.7 cm at 10 µg/g for *Adathoda visca* extracts, the next highest zone of inhibition was observed for extracts from *Abutilon indicum*,1.3 cm at 6 µg/g, 2.2 cm at 8 µg/g and 2.5 cm at 10 µg/g, *Datura stramonium* extracts showed inhibition zones of 1.1 cm at 6 µg/g, 2.3 cm at 8 µg/g and 2.4 cm at 10 µg/g. The inhibitory zones for *Lantana camara* extracts were 1.1 cm at 6 µg/g, 2.1 cm at 8 µg/g and 2.2 cm at 10 µg/g. *Tridax procumbens* showed minor zone of inhibition as 1.0 cm at 6 µg/g, 1.1 cm at 8 µg/g, and 1.2 cm at 10 µg/g. Thus, *Adathoda visca* extracts have potent antifungal activities against *Candida albicans*.



(Given values are average of triplicate i.e. n=3),

Figure 1: Anti-fungal activity of five plant extracts (6, 8, and 10 µg/g) aganist *Candida albicans*.

The antifungal activity of the alkaloids fraction of five different plants was determined against *Trichoderma harzianum*, (Figure 2). The zone of inhibition was highest as 1.8 cm at 6 µg/g, 2.1 cm at 8 µg/g, and 3.0 cm at 10 µg/g for *Adathoda visca* extracts, the next highest zone of inhibition was observed for extracts from *Abutilon indicum*,1.4 cm at 6 µg/g, 2.0 cm at 8 µg/g and 2.5 cm at 10 µg/g, *Datura stramonium* extracts showed inhibition zones of 1.2

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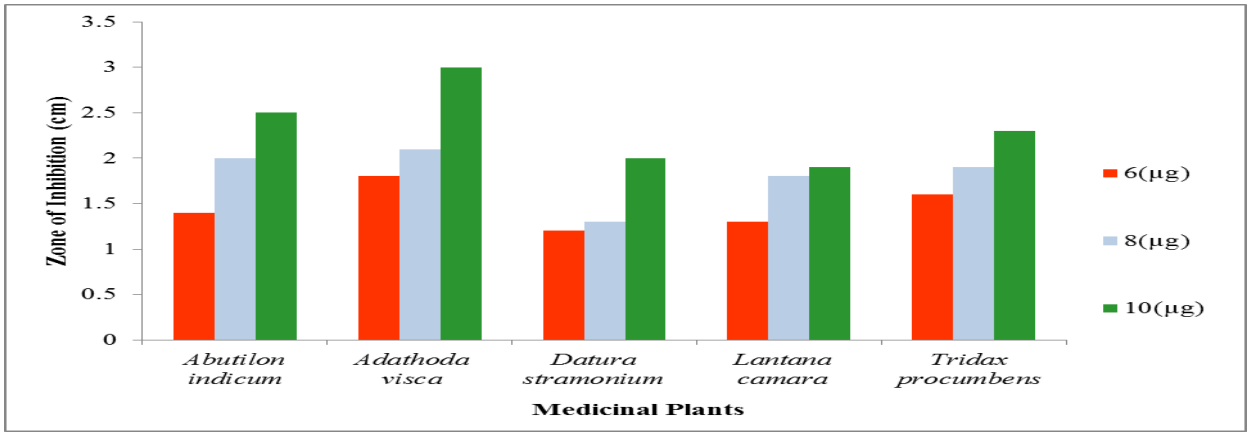
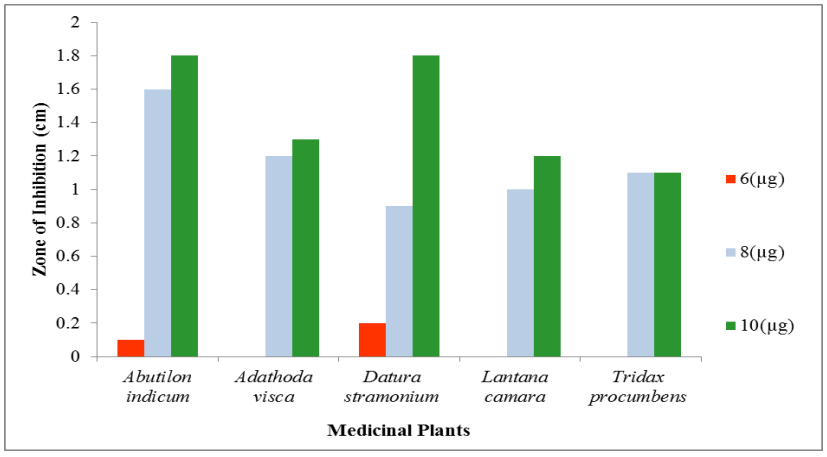
cm at 6 µg/g, 1.3 cm at 8 µg/g and 2.0 cm at 10 µg/g. The inhibitory zones for *Lantana camara* extracts were 1.3 cm at 6 µg/g, 1.8 cm at 8 µg/g and 1.9 cm at 10 µg/g. *Tridax procumbens* showed minor zone of inhibition as 1.6 cm at 6 µg/g, 1.9 cm at 8 µg/g, and 2.3 cm at 10 µg/g. Thus, *Adathoda visca* extracts have potent antifungal activities against *Trichoderma harzianum*.



(Given values are average of triplicate i.e. n=3),

Figure 2: The antifungal activity of the alkaloids fraction of five different plants was determined against *Trichoderma harzianum*.

The antifungal activity of the alkaloids fraction of five different plants was determined against *Aspergillus niger*, (Figure 3). The zone of inhibition was highest as 0.1 cm at 6 µg/g, 1.6 cm at 8 µg/g, and 1.8 cm at 10 µg/g for *Abutilon indicum* extracts, the next highest zone of inhibition was observed for extracts from *Datura stramonium* ,0.2 cm at 6 µg/g, 0.9 cm at 8 µg/g and 1.8 cm at 10 µg/g, *Adathoda visca* extracts showed inhibition zones of 0.0 cm at 6 µg/g, 1.2 cm at 8 µg/g and 1.3 cm at 10 µg/g. The inhibitory zones for *Lantana camara* extracts were 0.0 cm at 6 µg/g, 1.0 cm at 8 µg/g and 1.2 cm at 10 µg/g. *Tridax procumbens* showed zone of inhibition as 0.0 cm at 6 µg/g, 1.0 cm at 8 µg/g, and 1.1 cm at 10 µg/g. Thus, *Abutilon indicum and Datura stramonium* extracts have potent antifungal activities against *Aspergillus niger* and very less inhibitory zones identified in *Adathoda visca, Lantana camara* and *Tridax procumbens.*



(Given values are average of triplicate i.e. n=3),

Figure 3: The antifungal activity of the alkaloids fraction of five different plants was determined against *Aspergillus niger*.

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4. CONCLUSION

The results obtained from this work showed that plant extracts of *Adathoda visca, and Datura stramoniumLantana camara* and *Tridax procumbens.*, medicinal plants screened exhibit antifungal effects against *Aspergillus niger, Candida albicans* and *Trichoderma harzianum*.offer effective bioactive compounds for growth inhibition of the fungi. Even at low concentrations, these species showed antifungal activity nearly equal to that of the commercial fungicide used as a positive control. This study paves the way for the development of bioactive natural products with phytosanitary applications, with the added benefits of an environmentally safe and economically viable product. Further studies are needed to determine the chemical identity of the bioactive compounds responsible for the observed antifungal activity. Natural plant-derived fungicides may be a source of new alternative active compounds, in particular with antifungal activity. The high proportion of active extracts in the assayed species, selected according to available ethnobotanical data, corroborates the validity of this approach for the selection of plant species in the search for a specific activity.

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REVIEW ON PHARMACOLOGICAL PROPERTIES OF *PITHECELLOBIUM DULCE* TREE

Surendra A. S.[1*], Siddalinga Murthy K. R.[2], Malini M. R[1].

1. Department of Biochemistry, Ramaiah college of Arts, Science and Commerce, MSRIT post, Bengaluru – 560 054, India.
2. Department of Biochemistry, Jnana Bharathi Campus, Bangalore University, Bengaluru-560 056, India.

ABSTRACT: *Pithecellobium dulce* is a widespread tropical leguminous plant found in abundance in India, having several bioactive components serving mankind therapeutically since the ancient times. Almost all important parts of this plant like seeds, leaves and fruits are edible and non-toxic for human consumption and treatment of several ailments. A detail review on the significance of each of these parts viewing its pharmacological properties such as anti diabetic, antioxidant, antifungal and anti-inflammatory properties help in providing an in depth knowledge of this plant and its potential in applied medical field. This paper provides an insight to *Pithecellobium dulce* to establish its pharmacological potentials and widespread biochemical spectrum.

KEY WORDS: *Pithecellobium dulce,* anti diabetic, hepatoprotective, antioxidant, anti-inflammatory.

[*]**Corresponding Author: Mr. Surendra A. S.**
Assistant Professor, Department of Biochemistry, Ramaiah College of Arts, Science and Commerce, MSRIT Post, MSR nagar, Bengaluru – 560054.
E mail: suri.jain@gmail.com, Phone: +91-9448705669

Introduction

Plant contributes to a very significant fraction in the healthcare. About 70% of them are discovered in tropical areas and rest (30%) are discovered in the temperate and alpine areas. It is expected that not less than half of the medicine under clinical trial owe their origin to plants. In India, traditional medicine alone uses around 7000 plant species [1]. We are thus, highly dependent on these plant species for the maintenance of good health [2].

Many of these plants contain properties such as antimicrobial, anti-cancer, anti-diabetic, anti-atherosclerosis, immunomodulatory and even reno-protection and hepato-protective effects. As antioxidants have beneficial effects, particularly natural antioxidants, in the treatment and prevention of diseases, recently there has been a considerable interest in finding natural antioxidants from plant sources.

The *Leguminosae* constitutes to one of the largest families of angiosperms with over 12000 species grouped into 600 genera. This family is widely divided in to three sub families namely *Caesalpinioideae, Papilionoideae,* and *Mimosoideae. Pithecellobium* is one of these genus belonging to the subfamily Mimosoideae and is distributed widely in the tropics, mainly in Asia and America [3]. There are about 10 species of *Pithecellobium* genus *P. clypearia, P. dulce, P. jiringa, P. parviflorum, P. monadelphum, P. globosum, P. unguiscati, P. arboreum, P. flexicaule,* and *P. mart* which is distributed abundantly in India [4].

Pithecellobium dulce is popular for its edible fruits and for its traditional benefits in curing several ailments. This tree has been commonly used for fencing and tanning, as fodder for feed and pods for food. Infusions of different parts of *Pithecellobium dulce* have been

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traditionally used to treat diseases for example; skin of the stem is used for dysentery, leaves for intestinal disorders and seeds for ulcers [5, 6]. From simple aches of ear, tooth to helping in peptic ulcer cure, this plant has shown considerable relief.This therapeutic advantage comes from the novel metabolites this tree possesses. Preliminary phytochemical analysis of leaf extracts of this tree (benzene, chloroform, acetone and methanol) showed the presence of alkaloids, anthraquinones, cardiac glycosides, proteins, tannins, terpenoids, and sugars. Phytochemicals like flavonoids, terpenoids and steroids have received considerable attention due to their diverse pharmacological properties including antibacterial, antifungal and antioxidant activities. The tree is also reported to possess abortifacient [7], anti-inflammatory [8], anti venom [9], protease inhibitory [10], spermicidal [11], antimicrobial and antitubercular activity [12, 13]. Although the existing chemical, pharmacological and clinical literature on the plant is impressive, there are several aspects that remain unexplored.

Taxonomy of the plant *Pithecellobium dulce*:

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Fabales

Family: Fabaceae

Subfamily: Mimosoideae

Genus: Pithecellobium

Species: *Pithecellobium dulce*

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Figure 1: *Pithecellobium dulce* Tree (a) and its fruits (b)

2. Significance of *Pithecellobium dulce* Tree:

Almost all parts of *Pithecellobium dulce* tree [Fig. 1(a)] have shown the presence of novel metabolites which could contribute therapeutic needs in some way or the other. Many researchers are interested in its leaves, bark, roots, seeds, etc to explore the diverse phytochemistry which could determine its potential in the medical and industrial field.

2.1 Leaves:

The leaves are abruptly bipinnate with a single pair of pinnae per leaf and two pairs of leaflets per pinna, i.e. 4 leaflets in all per leaf. The leaves of *Pithecellobium dulce* have been identified with significant compounds like octacosanol, quercetin, α-spinasterol, cyclitol, dulcitol, kaempferol-3- rhamnoside, and afzelin [14, 15]. The qualities exhibited by these compounds help in the cure of peptic ulcer, toothache, leprosy, intestinal disorders, ear ache, emollient, larvicidal, and abortifacient in several folk medicines [16]. Recently, the leaves of the tree have reported to contain the insulin-like content that to aid diabetes [17]. These leaves also exhibit antifungal and antibacterial activities [18]. They also promote free radical scavenging and assist anti mycobacterial activity [19, 16].

2.2 Bark:

Bark is usually multiple-stemmed, sometimes forming only a bush, but often forming a branchy tree with an irregular rounded crown and flexuous and pendulous branches. It is used as an astringent, in cases of dysentery and febrifuge. It has been reported to abate symptoms of dermatitis and eye inflammation [20].

2.3 Seeds:

The pods open along both sides to reveal 8-12 seeds which persist after the pods open, attached by the fleshy white, pale pink or occasionally red, aril. The seeds are shiny black, compressed, lentiform, 7-13 x 6-11 x 2-4 mm in size [21]. A steroid saponin, lipids, phospholipids, glycosides, glycolipids and polysaccharides have been reported from the seeds [22].

2.4 Fruit:

The fruit extract was found to be rich in phenolic compounds and revealed the presence of flavonoids such as quercitrin, rutin, kaempferol, naringin and daidzein [20]. These fruits [Fig. 1(b)] are often consumed regularly as a dietary supplement for its high nutritive and medicinal value. This edible fruit is well known traditionally to combat gastric problems and found to be highly suitable for human consumption on daily basis. The fruit has abundant concentration of phenolic compounds too. The pulp of Manila Tamarind is traditional remedy

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for gum ailments, toothache, and haemorrhage [23].

2.5 Roots:

Estrogenic activity was proved by isolated isoflavonoids from the root of the plant [20].

3. Pharmacological Applications

3.1 Anti diabetic Property

The traditional system of Indian medicine, has prescribed several formulations of plant extracts which serve as the drug of choice which aids to fight as anti diabetic, hepatoprotective, anti ulcerative, and lipid-lowering agents [20].

Tests like amylase inhibitory activity was conducted on *Pithecellobium dulce* fruit peel for its anti diabetic activity. The IC_{50} values of this tree compounds was found to be 80.9% and 56.5% at 1 mg/ml. The findings indicated *Pithecellobium dulce fruit* peel to have inbuilt strong hypoglycemic effect which could serveas an adjunct in the control of diabetes mellitus [23]. Another study conducted with 200 mg/kg and 400 mg/kg of aqueous and ethanolic extract of *Pithecellobium dulce* promoted significant ($p < 0.05$) anti diabetic activity. This study on albino mice observed a significant reduction in blood glucose level on 11^{th} day when compared to diabetic control group. There was drastic rise in blood glucose, triglyceride, and total cholesterol level with decline in liver and muscle glycogen levels in the diabetic control group when compared to normal control group. This stated that these concentrations of *Pithecellobium dulce* possessed anti diabetic and hypolipidemic activity [24].

Tests conducted on methanol extract of *Pithecellobium dulce* against maltase and sucrase enzymes was found have IC_{50} values of 10.32±1.52 and 2.84±0.96 mg/ml respectively and against pancreatic α-amylase had 16.75±1.81 mg/ml. These results stated the enzyme inhibitory activity of this tree which could be endorsed to the availability of oleanolic acid and triterpenoid, thus justifying its traditional use in the management of diabetes [25].

Many scientists have been successful in giving a detailed report on enzymatic levels that pertain to diabetic control by this tree. They have observed significant reduction in the levels of blood glucose, glycosylated haemoglobin, urea and creatinine along with normalization of the altered levels of serum aminotransferases and alkaline phosphatase. This was supported by a decline of plasma protein, plasma insulin and haemoglobin levels to nearing normalization. This validated *Pithecellobium dulce* as nontoxic and its possession of anti diabetic nature.

3.2 Hepatoprotective Activity

Hepatoprotective nature was studied by inducing hepatic damage through alcohol and paracetamol treatments in rats which resulted in enlargement of liver observed through increase in liver weight and volume. Aqueous and ethanolic extracts of *Pithecellobium dulce* fruits was induced at different concentrations to albino mice to estimate the acute toxicity test. It was observed that study for 8 days, no mortality was observed in group with 2000 mg/kg injection of this fruit [26]. Another study involved action of aqueous extracts of *Pithecellobium dulce* in CCl_4-induced hepatic disorders. It was noted that CCl_4 administration lead to hepatic oxidative stress and cellular death mostly via the necrotic pathway and the introduction of aqueous extract of this tree prevented the toxin-induced hepatic damage in a large way [27].

Other parameters which supported the action of *Pithecellobium dulce* tree were histological and ultrastructurally proven and detailed study showed the normalization of the mitochondrial size, regeneration of rough endoplasmic reticulum, improved antioxidant competence in hepatic cells [28].

3.3 Antifungal property

Plants based antimicrobials could significantly help with the treatment of infectious diseases

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in addition to mitigating several of the side effects associated with synthetic antimicrobials.

A protein of an apparent molecular mass of 14.4 kDa with antifungal activity was isolated from the seeds of *Pithecellobium dulce* using extraction with 100 mM Tris-HCl buffer (pH=8.0), precipitation with 80% NH_4SO_4 and bioassay purification through Resource Q anion exchange chromatography and Superdex 200 gel filtration chromatography. The purified protein showed lot of resemblance to chicken egg white lysozyme. This protein apart from exhibiting antifungal activity towards *Macrophomina phaseolina* showed high thermal stability of up to 80 °C for 15 min (at pH=8.0) [29].

Another study exhibited the pulp extract of *Pithecellobium dulce* showing bactericidal and fungicidal activity with the Minimum fungicidal concentration (MFC) value of pod pulp extract against fungal strains ranging from 1 mg to 7 mg which were comparable standard Amphotericin B [30].

Acetone and methanol fractions of *Pithecellobium dulce* bark showed remarkable antifungal activity against *Aspergillus niger*. This was explained identifying bioactive compounds having significant antimicrobial activity [31].

Hexane dichloromethane and methanol-water extracts of the seed powder of this tree showed good antifungal activity towards *Fusarium oxysporum* and *Rhizopus stolonifer* while *Penicillium digitatum* was less sensitive towards these extracts. Several triterpene saponins isolated from the methanol-water extracts prevented in vitro mycelial growth of *Rhizopus stolonifer* and *Colletotrichum gloeosporioides* respectively [32].

3.4 Antioxidant Property

Oxidative stress aroused from free radicals formations and reactive oxygen species are associated with several disorders. Many investigations are carried out to trace out natural antioxidants from plant origin. Many theories support that plant extracts containing high phenolic compounds are strong antioxidant agents. The aqueous and methanol extract of *Pithecellobium dulce* seeds possessed good dose dependant free radical scavenging activity with high amount of total phenolic content of 1.31 ± 0.006 and 1.74 ± 0.003 mg gallic acid equivalents/g of extract powder respectively [33].

Another study suggested that total antioxidant activity (IC_{50} values) in DPPH assay conducted taking methanol extract of bark (MB), acetone extract of bark (AB), methanol extract of leaf (ML) and acetone extract of leaf (AL) showed 150.23 ± 2.8, 16.83 ± 0.38, 250.32 ± 4.8 and 18.30 ± 0.43 µg/ml respectively. This proved that the methanol and 70% acetone extracts of *Pithecellobium dulce* bark and leaves are best natural antioxidants and some extent validated its medicinal potential [34].

3.5 Anti-inflammatory property

The methanol extract of leaves of *Pithecellobium dulce* Benth was subjected to rat paw oedema test to establish the anti-inflammatory activity at varied concentrations. It was observed that maximum activity was noted at 3 hours in 200 and 400 mg/kg extracts proving that the extracts exhibited significant anti-inflammatory activity [35].

Another study conducted In-vitro anti-inflammatory activity through albumin denaturation assay and membrane stabilization assay. It was found that ethanol extracts of *Pithecellobium dulce* leaves showed good anti-inflammatory model in both the assays. Maximum membrane stabilization of *Pithecellobium dulce* was found at 73.85 % at a dose of 1000µg/0.5ml and that of protein denaturation was found to be 86.23% at a dose of 250µg/ml with regards to standards in anti-inflammatory activity [36].

3.6 Ovicidal and larvicidal property

Pithecellobium dulce was tested for its larvicidal property along with ovicidal activity as crude extracts with hexane, benzene, ethyl acetate and chloroform against *Culex quinquefasciatus and filariasis* vector mosquito. It was noted that methanol extracts of leaf

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and seeds were most effective with 164.12 mg/L, 214.29 mg/L, 289.34 mg/L and 410.18 mg/L against the larvae after a 24 hour period. Least activity was shown by hexane extracts. Here, nearly 100% mortality rate was noted with 500mg/L of leaf and 750 mg/L for seed extracts of this plant [37]. Similar study of seeds also inferred that they inhibit *Anopheles stephensi* and *Aedes aegypti* larvae growth [38].

3.7 Cardio protective activities

Pithecellobium dulce fruit extract of ethanol and water activated the marker enzymes including serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), cardiac marker enzymes such as creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) in isoproterenol (ISO) induced rats [39]. Bhavani et al., [40] added that both fruit and flower extracts of this plant showed comparable activities of enzymes obtained from rats with cardiac damage induced by isoproterenol.

3.8 Anticonvulsant activities

Epilepsy is a disorder characterized with recurrence of seizures accompanied by loss or disturbance of state of mind, with or not necessarily with unusual body movements indicated by an abnormal and higher electroencephalogram discharge. Anticonvulsant activity of flavonoid fraction of the leaf of *Pithecellobium dulce* was studied on the subcutaneous Pentylenetetrazole (PTZ) and Maximal electroshock test (MES) models in rats, showed notable decline in hindleg extension and convulsion dose [41]. A similar experiment detected ethanol and aqueous *Pithecellobium dulce* leaf extract to be anticonvulsant naturally. Maximal electroshock-induced seizure (MES) in rats exhibited significant reduction of extension phase time with maximum reduction in aqueous extract [42].

3.9 Other activities

Megala and Geetha, [43] noted gastro protective effect possessed by hydro alcoholic fruit extract of *Pithecellobium dulce* through activated of enzymes such as catalase, glutathione peroxidise, glutathione reductase, superoxide dismutase and myeloperoxidase. However, Mule et al., [24] stated aqueous and ethanol extracts could not produce any anticonvulsant activity by pentylenetetrazole method on mice. In spite of this, aqueous fruit extract did protect the murine liver against CCl_4-induced oxidative impairments suggesting its anti oxidative nature. According to Sugumaran et al., [42] study on aqueous and alcoholic leaf extracts of leaves, the locomotor activity and CNS depressant activity of leaf was examined using actophotometer in albino mice.

4. CONCLUSION

Pithecellobium dulce has proven to be beneficiary in several aspects of Indian traditional system of medicine. The seeds are known to be eaten raw or added in curries and the seed oil apart from being edible is used for soap manufacture. The bark having nearly 37% tannins, has several uses like astringent in dysentery, febrifuge, is also useful in dermatitis and eye inflammation. Polyphenols from bark extract of *Pithecellobium dulce* was also reported for their anti venomous activity.The *Pithecellobium dulce* fruits have shown good anti-inflammatory activity due to saponin fraction, free radical scavenging, H+, K+-ATPase inhibition, gastro protective and hepato protective effect. Presence of steroids, saponins, lipids, phospholipids, glycosides, glycolipids and polysaccharides has been reported in the seeds. The many pharmacological activities exhibited by the plant shows the presence wide range of bioactive compounds in the different parts of the plant. The phytochemicals of the plant are found to be remedy for many ailments and deeper understanding of molecular mechanism is required for potential drug development.

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MOLECULAR DOCKING STUDIES REVEALS ERIODICTYOL AS A POTENTIAL CARDIO PROTECTIVE AGENT

Preethi Rajesh[1], Vivek Chandramohan[2], Manju V[3]*
[1]School of Sciences, Garden City University, Bangalore.
Preethi.rajesh@gardencity.university
[2]Department of Biotechnology, Siddaganga Institute of Technology, Tumkuru
vivek@sit.ac.in
[3]Department of Biochemistry, Periyar University, Salem
manjucb11@gmail.com

ABSTRACT: Myocardial infarction is the most important form of Ischemic Heart Disease (IHD). Cardiovascular epidemiology studies in India indicate that it has become an important public health problem in India and has reached epidemic levels. The present study was focused on to elucidate the interaction of the flavonoid eriodictyol with various cardiac membrane receptors by molecular docking studies. The cardio protective potency of the flavonoid was evaluated by with respect to the stability of interaction in terms of hydrogen bond, electrostatic energies, Binding affinity, interaction of amino acid residues of eriodictyol with the membrane proteins. The binding property and stability of various cardiac receptors were studied with the ligand molecule eriodictyol. Molecular Docking results suggest that the interaction and stability of eriodictyol was optimum with GPCR protein and possibly can be a potent cardiovascular agent.

KEYWORD: Myocardial infarction, molecular docking, GPCR.

Corresponding Author: Dr. V Manju*, MPhil, Ph. D.
Department of Biochemistry, Periyar University, Salem.

1. INTRODUCTION

Cardiovascular disease is the leading cause of death and disability throughout the world [1]. Myocardial infarction is the most important form of Ischemic Heart Disease (IHD), in which ischemia causes the death of heart muscles, characterized by necrotic cell death because of the breakdown of cellular energy metabolism. It is estimated that mortalities due to cardiovascular diseases will increase by 15% in developed countries like United States [2], 77% in China and a tremendous 106% in other Asian countries [3]. The annual incidence of acute myocardial infarction for persons aged 30–69 years is estimated by the British Heart Foundation at 0.6% for men and at 0.1% for women [4]. Under the leadership of World Health Organization all the member nations which includes 194 countries has agreed to work on a common platform evoking global mechanisms to reduce the CVD burden by 25% by 2025 by evolving " Global action plan for the prevention and control of NCDs 2013 – 2020" by focusing directly on the prevention and control of cardiovascular diseases.

G-protein-coupled receptors (GPCRs) are transmembrane receptors that is one of the largest classes of receptors found in the cardiac myocytes [5], GPCR signaling pathways accounts for a majority of drug targeted therapy for cardiovascular diseases [6] An understanding of the GPCR signaling pathways , and the interaction of ligand molecule with these membrane receptors is critical in the drug designing and discovery against cardiovascular diseases. Another class of membrane receptors in cardiac myocytes are receptor tyrosine kinase.

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Protein tyrosine kinases (PTKs) phosphorylate tyrosine residues within proteins. The RTK family includes epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor receptor (FGFR). RTKs play important roles in cardiac myocytes and are principally involved in initiating signalling pathways that control apoptosis, cell growth, proliferation, and metabolism [7]. Signalling through receptor tyrosine kinases promotes cellular events that protect against cardiomyopathy[8].

Eriodictyol is a flavanone , 2-(3,4-dihydroxy phenyl)-5,7-dihydroxy-2,3-dihydro-4H-chromen-4-one (Figure 1) extracted from *Yerba Santa* [9].

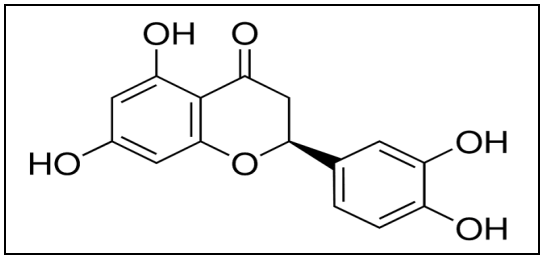

Figure 1: Structure of eriodictyol, its molecular formula is $C_{15}H_{12}O_6$,
Molecular weight is 288.5 g/mole

Molecular docking is computer-assisted drug design and is a key tool in structural molecular biology. The ligand-protein docking enables to predict the predominant binding model of a ligand with a protein of known three-dimensional structure. Successful docking methods search high-dimensional spaces effectively and use a scoring function that correctly ranks candidate dockings.

2. MATERIALS AND METHODS

Molecular docking studies were carried out using the Lead IT software which uses flexible docking approach i.e. it considers ligand flexibility by changing the conformations of the ligand in the active site while making the protein rigid [10]. The images were visualized using Accelrys Discovery studio visualizer and DS visualizer Active X control. The 3D crystal structure of the protein receptor under study was retrieved from Protein Data Bank with query ID 2YDO [11], 2Z8C [12], 3NY8 [13] and 3VHK [14]. Energy minimization before docking process removes undesirable interaction among atoms of the ligand and enabling a stable confirmation to initiate docking with target membrane receptors by energy minimization protocol [15]. The binding property and stability of these four receptors were studied with the ligand molecule eriodictyol.

3. RESULTS AND DISCUSSION

Figure 2 gives the interaction of the membrane receptor 2YDO, G-protein-coupled receptors (GPCRs) with eriodictyol. The major amino acids involved in the interaction were ALA 63, ILE 66, VAL 84, LEU 85, THR 88, PHE 168, MET 177, TRP 246, LEU 249, HIS 250, ASN 253, ILE 274, SER 277, and HIS 278. There are two hydrogen bonds favoring the interaction, contributed by the amino acid ALA 63 and THR 88.

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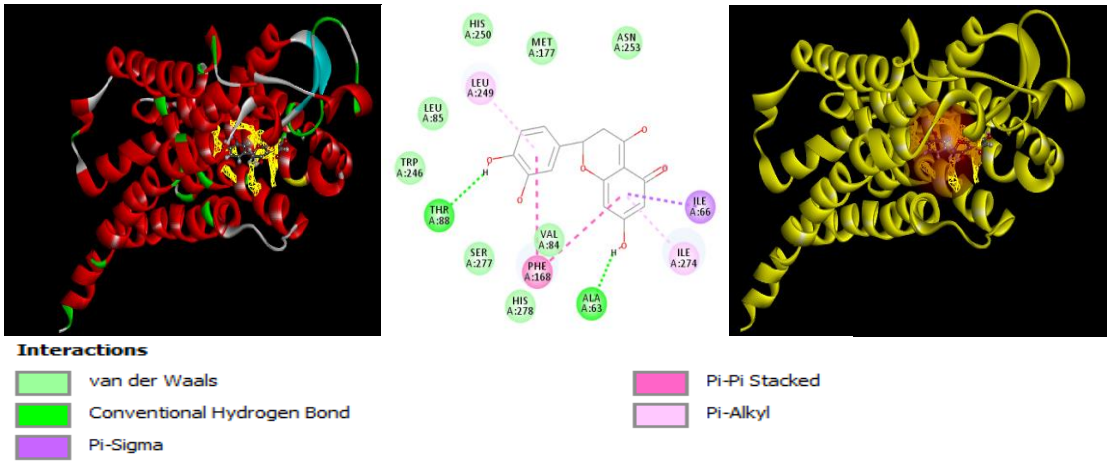

Figure 2: Molecular docking of 2YDO Adenosine Receptor GPCR with the ligand molecule eriodictyol

Figure 3 gives the interaction of the membrane receptor 2Z8C, protein tyrosine kinases with eriodictyol. The molecular docking studies show that the major amino acids which favor the interaction were LEU 998, GLU 1001, GLN 1004, GLY 1008, MET 1009, VAL 1010, TYR 1011, VAL 1029, LYS 1030, THR 1031, VAL 1032, ASN 1033, GLU 1034, PRO 1071, LEU 1073. The three hydrogen bonds formed during this interaction is contributed by GLU 1001, VAL 1010 and VAL 1029.

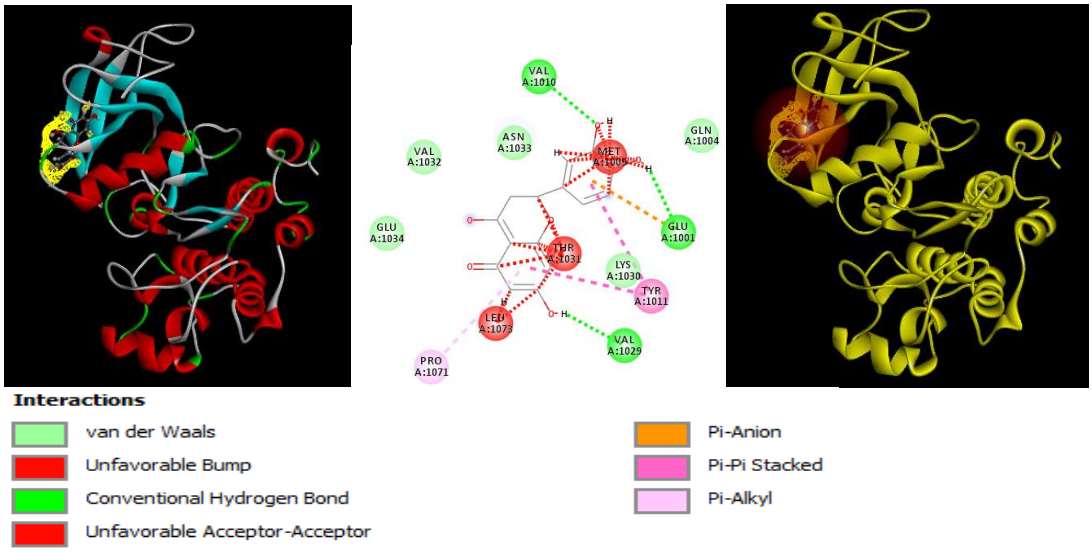

Figure 3: Molecular docking of 2Z8C Insulin Growth Factor Receptor Tyrosine Kinase with the ligand molecule eriodictyol

Figure 4 gives the interaction of the membrane receptor 3NY8, G-protein-coupled receptors with eriodictyol. Docking studies show that the amino acids involved in the interaction were THR 110, ASP 113, VAL 114, PHE 193, THR 195, TYR 199, ALA 200, SER 203, SER 204, TRP 286, PHE 289, PHE 290, ASN 293, TYR 308, and ASN 312. This interaction is stabilized by six hydrogen bonds contributed by four amino acid namely ASP 113, TYR 199, ALA 200 and ASN 312.

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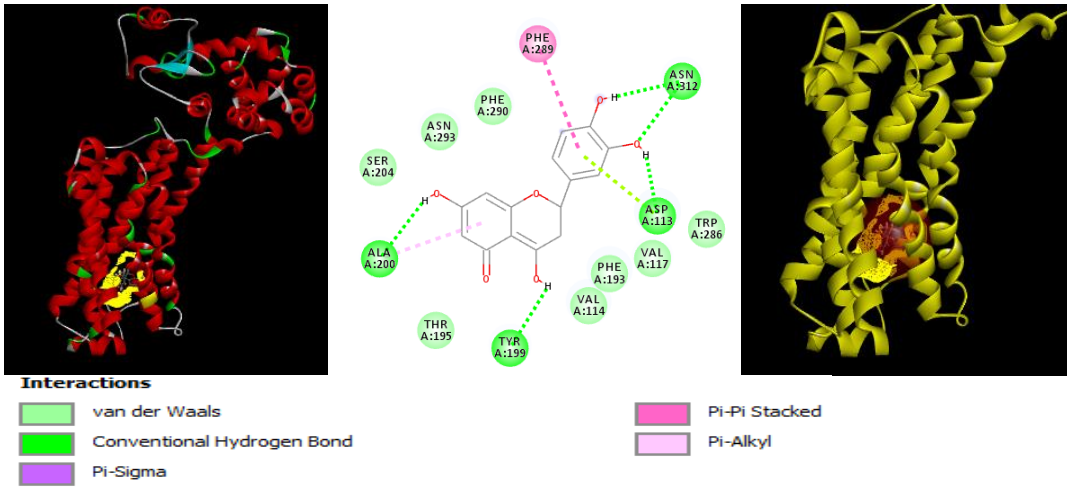

Figure 4: Molecular docking of 3NY8 Adrenergic Receptor GPCR Protein with the ligand molecule eriodictyol

Figure 5 gives the interaction of the membrane receptor 3VHK, protein tyrosine kinases with eriodictyol. The study reveals that the amino acids involved
in the interaction were LYS 868, LEU 882, GLU 885, LEU 886, ILE 888,
LEU 889, ILE 892, VAL 898, VAL 899, ASN 900, LEU 901, VAL 914, VAL 916, GLU 917, LEU 1019, HIS 1026, ASN 1033, LEU 1035, LYS 1043, ILE 1044, CYS 1045, ASP 1046, PHE 1047 and GLY 1048.The amino acids VAL 899, HIS 1026 and CYS 1045 stabilizes this interaction by the formation three hydrogen bonds.

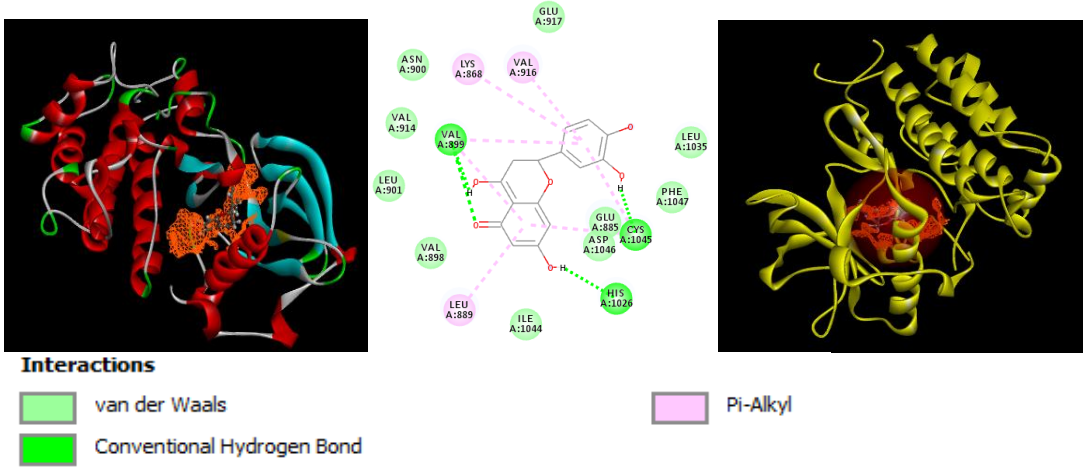

Figure 5: Molecular docking of 3VHK Vascular Endothelial Growth Factor Receptor Tyrosine Kinase with the ligand molecule eriodictyol

The result obtained in terms of CHARMm energy, angle energy, dihedral energy, electrostatic energy, potential energy, van der Waal's energy and bond energy is tabulated in Table 1. It is found that NY8 Adrenergic Receptor GPCR protein has a value of CHARMm energy -25,656.1 and bond energy 307.369, 2 Z8C IGFR RTK has a CHARMm energy -19,206.5 and bond energy 201.301, 3 VHK VEGF RTK has a CHARMm energy -18551.1 and bond energy 200.783 and 2YDO Adenosine Receptor GPCR has a value of CHARMm energy as -16,359 and bond energy 195.809. Collating the data and its analysis shows that interaction and stability of eriodictyol were maximum with 3NY8 Adrenergic Receptor GPCR protein.

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Receptors coupled to heterotrimeric GTP-binding proteins (G proteins) are the largest group of integral membrane receptors involved in the transmission of signals from the extracellular environment to the cytoplasm [16].The interaction of flavonoid eriodictyol with GPCR protein can cause a conformational change in the GPCR, leading to its activation [17]. PI3K can be activated by G protein coupled receptors (GPCR), Ras proteins and by various signalling molecules [18].The phosphorylated PI 3K(PI (3,4,5) p3) can directly bind to Akt activating it by its phosphorylation,and translocates it into cytosol and nucleus [19]. Akt can also been activated by inflammation, DNA damage and also by an increase in cellular Ca^{2+} concentration [20]. Activated Akt influences the activity of apoptotic proteins by direct

Table 1: Molecular docking report of eriodictyol with membrane receptors

Compound	CHARMm energy	Angle Energy	Dihedral Energy	Electrostatic Energy	Potential Energy	Van der Waals Energy	RMS Gradient	Bond Energy
2YDO Adenosine Receptor – GPCR	-16,359	1,068.01	975.822	-16,715.9	-8,085.39	-2,007.55	0.93659	195.809
3 NY8 – Adrenergic Receptor – GPCR	-25,656.1	1,463.99	1,333.98	-25,954.6	-25,656.1	-2,990.64	1.03052	307.369
2 Z8C – IGFR RTK	-19,206.5	1,117.89	1,107.53	-19,761	-19,206.5	-2,022.31	1.15078	201.301
3 VHK – VEGF RTK	-18551.1	1,053.83	873.931	-18,619.7	-18,551.1	-2,197.22	0.93792	200.783

phosphorylation or by the regulation of transcription [21]. The excessive production of reactive oxygen species and cytosolic calcium overload are the key factors in inducing the opening mitochondrial permeability transition pore leading to necrosis [22]. PI3k/Akt are important regulators of glycogen synthase kinase (GSK 3β) pathway. Increased phosphorylation of GSK 3β by PI3k/Akt inactivates GSK 3β limiting changes in mitochondrial permeability transition reducing apoptotic cell death [23]. In the present study it theoretically reveals that cardio protective effect of eriodictyol may be attributed to its interaction with GPCR protein and its role in activating the Akt expression as represented in Figure 6 and thereby exerting control on signaling molecules modulating mitochondrial mediated necrotic pathway.

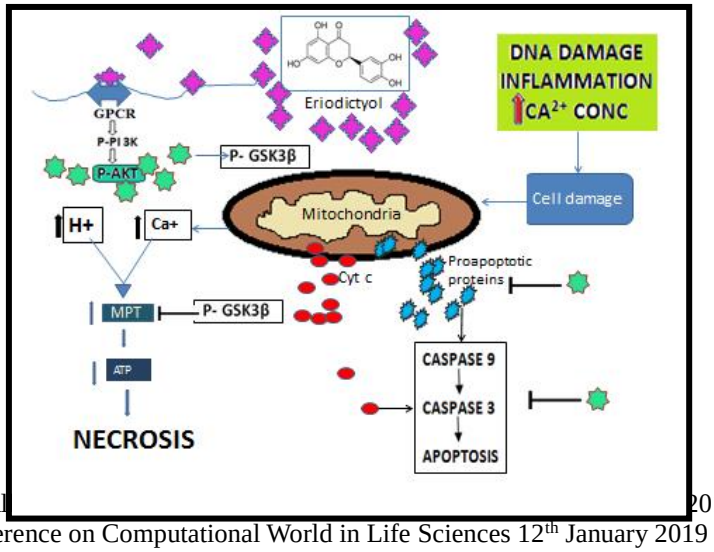

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Figure 6: Pictorial representation of the cardio-protective effect of eriodictyol.

4. CONCLUSION

Molecular Docking study reveals that Eriodictyol can interact with both GPCR and RTK proteins. The interaction is stronger and stable with GPCR protein when compared with RTK proteins. The eriodictyol can be a potential cardio protective agent considering its level of interaction with GPCR proteins. The integration of computational and experimental strategies has been of great value in the identification and development of novel promising compounds as valuable drugs.

CONFLICT OF INTEREST

None.

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MOLECULAR ASSESSMENT OF GENETIC STABILITY USING ISSR MARKER IN *IN VITRO* MICROPROPAGATED PLANTS OF *HYPERICUM MYSORENSE*

Shilpashree H P[1*] and Ravishankar RV[2]
[1]Department of Biotechnology, Acharya Bangalore B School, Bangalore-560091, India.
[2]Department of studies in Microbiology, University of Mysore, Mysore-570006, India.

ABSTRACT: Micropropagation is a technique which is used to obtain uniform *in vitro* regenerated plant material of significant medicinal, horticultural, ornamental and agricultural plants. However, it is necessary to authenticate the clonal uniformity of *in vitro* raised plants to confirm the reliability of the protocol for mass-propagation. DNA based molecular markers are more reproducible as compared to the cytological, morphological and protein markers as they have been long practiced for the identification of any genetic variations in tissue culture raised plantlets and other genetic diversity studies for plant identification. Moreover, these markers are highly reproducible, inherited, reliable, detectable in all tissues and easy to perform. We have standardized the protocol for *in vitro* plant regeneration of an important medicinal plant *Hypericum mysorense* [1]. In the present study, the genetic fidelity was assessed among the 14 *in vitro* plant regenerants of *H. mysorense* and compared with their mother plant using the ISSR marker. Screening with the 40 ISSR primers, 36 primers were able to produce scorable bands. A total of 4,470 bands were generated by using ISSR primers which give rise to monomorphic patterns across all 14 regenerants analyzed compared to mother plant. Thus, regenerated plantlets of *H. mysorense* could be used as a source for the production of essential bioactive compounds like rutin, quercitin, quercitrin and hyperoside.

KEY WORDS: Genetic fidelity, ISSR, PCR, micropropagation, medicinal plant.

[*]**Corresponding Author**:
Dr. Shilpashree H. P. Ph. D.
Department of Biotechnology, Acharya Bangalore B-School
Email Address: hpshilpashree16@gmail.com*

1. INTRODUCTION

In recent years, medicinal plants are the most important lifesaving drugs for majority of world's population. Plant based medicines plays an indispensable role in maintaining human health and combating several diseases. The identification of active principles and their molecular targets from traditional medicine provides an enormous opportunity for drug development. Micropropagation of these medicinal plants is widely used to produce bioactive compounds in herbal formulations and pharmaceutical industries. Using modern biotechnology, plants with particular chemical compositions can be mass-propagated and genetically improved for the extraction of pharmaceuticals. Although there has been significant progress in the use of plant tissue cultures technique and genetic transformation to investigate and alter pathways for the biosynthesis of target metabolites, there are many challenges involved in bringing plants from the laboratory to successful commercial cultivation (first REFERENCES[1] is in ABSTRACT) [2]. So, in micropropagation, one of the most crucial concern is to retain the genetic stability of *in vitro* propagating plant material. Hence a genetic fidelity assessment to obtain true to type plant material at an early stage of development is considered to be very useful in plant tissue culture technology [3].

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In vitro propagation system is used for mass multiplication of plantlets that are genetically similar and phenotypically uniform, or else the advantage of desirable characters in selected clones may not be succeeded. Several methodologies have been useful for identifying variation among *in vitro* regenerated plants. They may be phenotypic variations, karyotypic change in metaphase chromosomes, morphological descriptions and phytochemical content [4-6].The disadvantage of these techniques is the insufficient number of informative detectable markers and dependence upon developmental processes during *in vitro* conditions [7]. In addition to this, initial variations may arise, mostly in response to the stress or hormonal difference under *in vitro* conditions. However, to study these different morphological and physiological changes involves extensive monitoring until maturity and are extremely sensitive to environmental distractions [8]. Therefore, more efficient detection tools like DNA markers must be used to assess the genetic fidelity of *in vitro* raised plants. Among the different DNA based molecular markers (like RFLP and RAPD) ISSR is considered to be a reliable technique for detecting variability among many *in vitro* raised commercially important plants [9].

Inter simple sequence repeats (ISSR) are regions in the genome flanked by microsatellite sequences. PCR amplification of these regions using a single primer yields multiple amplification products that can be used as a dominant multilocus marker system for the study of genetic variations. ISSR marker is widely useful because they do not require the knowledge of genomic sequences and also the protocol is relatively simple, rapid and cost effective [10]. It requires very small amount of template DNA and is convenient to analyze the data and highly reproducible. ISSR markers are known to be more reproducible than RAPD markers and they have been successfully applied to study the genetic information in many plants [11-13]. It permits detection of polymorphisms in microsatellites and inter microsatellites loci without previous knowledge of the DNA sequence. Also ISSR marker is informative about many loci and are suitable to differentiate closely related genotype variants and appropriate to obtain DNA fingerprinting profile of individual specie [14, 15].Based on these advantages the present study has selected ISSR as DNA molecular marker to detect any genetic variations in *in vitro* grown plant regenerants when compared to natural plants.

Hypericum mysorense is a pharmaceutically an important medicinal plant found in Western Ghats of Karnataka, India. Previous studies reports that extracts have shown strong antimicrobial, anticancer and antioxidant properties [16-18]. We have standardized the protocol for plant regeneration system by using both leaf and nodal segment as initial explants. Also, we have identified important flavonoids produced at high concentration in callus and *in vitro* regenerated plantlets compared to natural plants [1, 19]. After effective micropropagation system has been achieved using nodal segments and leaf as explants, it is necessary to check any somaclonal variations occurred during *in vitro* conditions. The aim of the present study was to analyze the genetic fidelity in *in vitro* grown plant regenerants of *Hypericum mysorense* to assure the true-to-type of the plantlets by using ISSR as DNA marker.

2. MATERIALS AND METHODS

2.1 Plant material and micropropagation

Hypericum mysorense plant samples were collected from Western Ghats of Karnataka in the month of July. The basic medium used for micropropagation was Murashige and Skoog medium (MS) containing 3% sucrose and solidified with 0.8 % agar. Nodal segments were

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used as initial explants. The MS medium containing 1.0mg/l 6-benzylaminopurine (BAP) of growth hormone showed maximum shoot initiation and multiplication. The highest root formation was observed in medium containing 0.5mg/l indole-3- acetic acid (IAA). Later, the *in vitro* raised plants were successfully acclimatized under greenhouse conditions [1]. These *in vitro* grown plantlets were used as samples for isolation of DNA for ISSR analysis.

2.2 DNA extraction

Total genomic DNA was isolated from the leaf tissues of the mother plant and regenerated plants using the CTAB (cetyl trimethyl ammonium bromide) method with few modifications [20]. 5.0 grams of leaf tissues were ground in liquid nitrogen and suspended in three volumes of 3% CTAB extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA and 1% β-mercaptoethanol). The suspension was incubated at 60^0C for 90 min, extracted with an equal volume of phenol: chloroform: isoamyl alcohol (24:24:1) and centrifuged at 5000 × g for 10 min, this step was repeated twice. The DNA was precipitated with a two-third volume of isopropanol at -22^0C for 2 h. The pellet was recovered and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then treated with RNAse at 37^0C for 1 h. The DNA was purified by phenol/chloroform extraction and ethanol precipitation in the presence of 0.3 M sodium acetate (pH 5.2). The pellet was dissolved in TE buffer and the DNA concentration was estimated in 0.8% agarose gel.

2.3 ISSR analysis

ISSR analysis of genomic DNA were carried out by using 40 primers. The PCR (polymerase Chain Reaction) was carried out in total volume of 20 µl reaction mixture containing 50 ng of genomic DNA, 1× assay buffer, 0.2 mM dNTPs, 1.75 mM $MgCl_2$, 0.2 µm primers and 0.1 U of Taq DNA Polymerase. The amplification was carried out in a DNA MJ Research Thermocycler (Germany) according to the programme: initial denaturation at 94^0C for 3 min, followed by 40 cycles of denaturation at 94^0C, annealing for 45 s (temperature varies for each primer from 42^0C to 48^0C) (Table-1), extension at 72^0C for 2 min and a final polymerization step at 72^0C for 10 min. The amplification products were resolved by using gel electrophoresis on 1.5% (w/v) agarose gel in 1x TBE buffer (Tris Borate, EDTA) at 75 V. The gels were stained with ethidium bromide solution. The amplified products were visualized and photographed under UV transilluminator. Molecular marker λDNA/EcoRI-HindIII was used to estimate the size of amplified products. PCR amplification with each primer was repeated twice to confirm reproducibility of the results. All the reagents used above were purchased from Sigma, Bangalore, India.

3. RESULTS AND DISCUSSION

During the last decade development in the PCR based techniques on DNA markers are used as powerful tools to validate the genetic fidelity of *in vitro* plants [21]. Since simple sequence repeat based primers target the fast evolving, hypervariable sequences, ISSR markers are appropriate to identify genetic variations among tissue culture raised plants [22, 23]. In the present study, screening with the 40 ISSR primers, 36 primers were able to produce 298 scorable bands, ranging in size from 250 bp to 3.8 kb. Primers 3, 11, 29 and 17 were not able to produce any amplification profiles. The number of bands for each primer varied from 2 to 8 with an average of 8.27 bands per ISSR primer. A total of 4,470 bands were generated by using ISSR primers which give rise to monomorphic patterns across all 14 regenerants analyzed compared to mother plant with 100% similarity, without showing any DNA polymorphism. An example of the monomorphic PCR amplification DNA profile obtained from ISSR primer HP-18 is shown in Figure-1. Similar supporting results has been reported in different commercially important micropropagated plants such as *Brassica oleracea* [24], *Populus tremuloides* [25], *Swertia chirayita* [26], *Bambusa balcoa* [27], *Simmond*

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siachinensis [28],*Lilium orientalis* [29], *Tylophora indica* [30], *Dendrocalamus strictus* [31]. However, ISSR fingerprinting has also been successful to identity somoclonal variation in *in vitro* plants of *Robinia ambigua* [32], *Amorphophallus rivieri* [33], *Phoenix dactylifera* [34]. There are numerous factors which are known to be associated to induce somaclonal variation such as, the *in vitro* plant development, duration, auxin and cytokinin concentration, media and other nutritional conditions, initial explants, *in vitro* conditions and stress, duration of plants growth and the species genotype [12, 35, 36]. Similarly Smith, [37] reviewed the factors contributing to variation and divided these into two, an intrinsic factor which largely depends on the genetic stability of the explant and an extrinsic factor depending on culture media and particularly growth regulators. Phytohormones, such as 2, 4-D, NAA, and KIN have been most frequently considered for inducing genetic variability [38, 39]. In the present study, the ISSR analysis strongly suggest that the successful micropropagation in the presence of growth regulators BA (1.0 mg/l) and IAA (0.5 mg/l) in MS medium ensures the maintenance of genomic integrity of regenerants during *in vitro* propagation, without inducing any somaclonal variability.

Also, some changes induced during *in vitro* culture may not be detectable under *ex vitro* conditions. Hence many researchers suggested to utilize the application of more than one DNA marker for better assessment of genetic fidelity of plants, as they will target different regions of the genome [40, 41].Nevertheless ISSR markers are capable to detect relatively low level of polymorphism compared to RAPD analysis on the same plants, based on the specific amplification of genome sequences by microsatellite primers. Therefore our results convinced that established *in vitro* plants derived from nodal segments as initial explant were clonally uniform and genetically stable. The DNA banding profile obtained from each ISSR primer confirmed that no significant genetic variations were detected with respect to tissue culture raised regenerants when compared to mother plants. Thus *H. mysorense* could be used for mass propagation and commercial production of important antimicrobial agents and flavonoids like rutin, quercitin, quercitrin and hyperoside [1].

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Table 1: List of ISSR primers used to screen micropropagated plants of *Hypericum mysorense.*

	ISSR Primers (5′-3′)	Annealing temperature (°C)
1.	CTCTCTCTCTCTCTCTAC	41
2.	CTCTCTCTCTCTCTCTGC	44
3.	CACACACACACAAC	39
4.	CACACACACACAGT	44
5.	CACACACACACAAG	45
6.	CACACACACACAGC	43
7.	GTGTGTGTGTGTGG	38
8.	GAGAGAGAGAGACC	39
9.	CACCACCACGCAC	47
10.	GAGGAGGAGGCCA	44
11.	CTCCTCCTCGCTCC	46
12.	GTGGTGGTGGCCCT	41
13.	GAGAGAGAGAGAGAGAT	40
14.	GACAGACAGACAGACA	40
15.	GATAGATAGATAGATA	38
16.	CCTACCTACCTACCTA	38
17.	ACACACACACACACACC	40
18.	ACACACACACACACACG	48
19.	GGGTGGGGTGGGGTG	41
20.	CTCTCTCTCTCTCTCTG	45
21.	CACACACACACACACAG	40
22.	TCTCTCTCTCTCTCTCA	39
23.	GGAGAGGAGAGGAGA	42
24.	CCCGTGTGTGTGT	45
25.	CCAGTGGTGGTGGTG	42
26.	AGAGAGAGAGAGAGAGC	44
27.	GAGAGAGAGAGAGAGAT	41
28.	GAGAGAGAGAGAGAGAC	38
29.	CTCTCTCTCTCTCTCTCTG	46
30.	CTCTCTCTCTCTCTCTCTG	42
31.	ACACACACACACACACC	42
32.	ACACACACACACACACG	48
33.	CTCTCTCTCTCTCTCTG	41
34.	CACACACACACACACAG	39
35.	GACAGACAGACAGACAC	45
36.	GGAGAGGAGAGGAGACA	40
37.	CCCGTGTGTGTGTGTTG	42
38.	CCAGTGGTGGTGGTGCC	47
39.	CCAGTGGTGGTGGTGAC	47
40.	CACCACCACGCA	39

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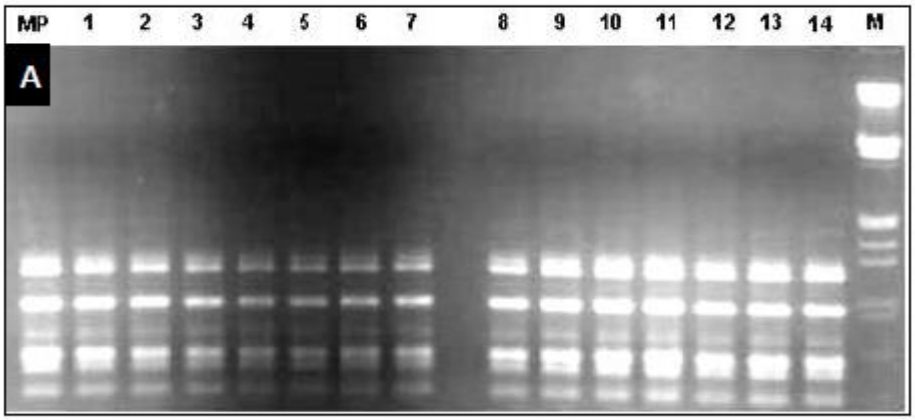

Figure 1: DNA banding profile obtained with the PCR amplification using ISSR primer 18. 1-14-*In vitro* plant DNA samples. M-DNA ladder (λ /DNA/EcoRI-Hind III), MP-Mother plant DNA

4. CONCLUSION

To the best of our knowledge, this is the first ever report on *H. mysorense* for the genetic fidelity assessment of successfully grown *in vitro* plants by using DNA based ISSR marker. The production of monomorphic bands by the mother plants and the *in vitro* raised plantlets against ISSR primers showed that there was almost no variability among the regenerated plantlets and the mother plant of *Hypericum mysorense* and hence can be concluded that the *in vitro* -raised plants avoided the genomic aberrations and did not lead to any somaclonal variation.

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STUDIES ON DECOLOURISATION OF DISTILLERY EFFLUENT USING MICROBIAL CONSORTIUM UNDER STATIC -BATCH MODE AND AGITATION - BATCH MODE

Ishwar chandra[1,2]*, N Ramesh[3], A Upadhyay[1]

[1]M Visvesvaraya Institute of Technology, Krishnadeveraya Nagar, Hunasamaranahalli Bangalore-562157,

[2]Department of Biotechnology, Ph.D scholar at Reva university,Bangalore.

[3]Dean, Faculty of Science and Technology & Training, Placement & Planning, REVA University, Bangalore.

ABSTRACT: The presence of melanoidins in Biomethanated distillery spent wash (BDSW) leads to water & land pollution due to its dark brown color and high COD levels. The objective of this study was physico-chemical and microbial population characterization of BDSW and to isolate a microbial consortium capable of decolourising and degrading BDSW. The colour removal from BDSW using bacterial consortium has been studied in static-batch mode and agitation-batch mode. Under agitation of 120 rpm, the bacterial consortium exhibited maximum decolourisation of 82.36% with minimal media compared to 38.55% in nutrient media within 24 hours of contact time at 37^0C. With sequential inoculation of the bacterial consortium to high strength BDSW (high COD), the drop in percent decolourisation was observed. Under static-batch mode the bacterial consortium showed maximum of 80% decolourisation at 37^0C after 17 days of contact time. Isolation and characterization of melanoidin degrading bacterial pure isolate from soil was performed which was capable of colour removal upto 77% under agitation of 120 rpm at 37^0C. When the isolate was subjected to high strength BDSW, the drop in percent decolourisation was observed.

KEYWORDS: *Decolourisation, distillery spent wash, Static condition, COD, microbial consortium*.

***Corresponding Author:** Ishwar Chandra

M Visvesvaraya Institute of Technology, Krishnadeveraya Nagar, Hunasamaranahalli Bangalore-562157, affiliated to Visvesvaraya Technological University, Belgaum

Research Scholar at Department of Biotechnology, REVA University, Banaglore
Email Address:ishwar.bt016@gmail.com

1. INTRODUCTION

Molasses based distilleries have gained too much attention recently and they are featured under the “Red Category” list as per the India Ministry of Environment and Forest (MoEF), since they are major environmental threat for sustainable development[1]. The waste water released from these industries is called as spent wash and it is capable of polluting recipient water and agricultural land by altering their chemical and biological properties [2-8]. The fundamental characteristics of discharge from these industries are, low pH (3.5- 5.0), high volume of 8-15 liter for each liter of ethanol [9], dark brown colour, high chemical oxygen demand (COD) (10), offensive odour and recalcitrant nature [10-11].

According to latest reports, there are around 397 operating distilleries in India producing

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more than 2.7 billion liters of alcohol and 40 billion liter of spent wash annually [12-13]. Therefore, treatment of this voluminous effluent is essential before its release to environment. Distilleries treat the spent wash either by physiochemical or biological methods. Physiochemical methods are highly effective in reducing COD, but methods like adsorption using activated carbon [14], flocculation & coagulation [15] generate secondary pollutants. Advanced methods like incineration in multiple effect evaporator suggested by AIDA & CPCB, India, advanced oxidation techniques, *viz.* Ultrasound and Ozone [16] ultrafilteration & reverse osmosis [17], and membrane technology [18] are cost intensive. Biological treatment involves sequential anaerobic digestion (Biomethanation) followed by aerobic treatment. This approach recovers methane gas and leaves behind Biomethanated distillery spent wash (BDSW), which is later treated in aerated lagoons. Compared to physiochemical methods, this method is less effective in reducing COD. But, because oflow maintenance cost and easy operation it is followed in most of the distilleries [19].

A major difficulty in biological treatment of spent wash is due to the presence of a polymer and a natural browning pigment called melanoidins [20-21]. The melanoidins have empirical formula of $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ with molecular weight of 5-40 KDa [22]. Melanoidinfrom BDSW have diverse structure & elemental composition [23-24], antioxidant nature [25], antimicrobial property [26]& toxic to the microbes used in spent wash treatment. Chiefly maillard product of sucrose–aspartic acid (SAA-MP) is the major colour imparting melanoidins in BDSW [27-28].

Despite of all these shortcomings, these methods are widely followed, and there is a need for a better technique. Due to strict instructions from CPCB India, distilleries are opting for zero-discharge by using the diluted spent wash for ancillary purpose, gardening, blending with concrete for construction etc. Since all these methods are concession, this scenario has created avenues for researcher to look for a suitable treatment method. Many authors have reported controlled use of BDSW for fertigation in top vegetables (Creepers), groundnuts, sugarcane cultivation, which minimizes the use of fertilizer and minerals but its long term usage is hazardous to soil properties [29-30]. All these existing solutions to the problem are not sustainable [31] and they fail to bring down the spent wash characteristics below the standards set by CPCB. Previous work has only focused on reduction of COD and they have failed to address the problem of colour. The present work is aimed at increasing the overall efficiency of the distillery spent wash decolourisation process by using a mixed microbial consortium.

2. MATERIALS ANDMETHODS

2.1 Medium and Organism

Physico-chemical & biological parameters like colour, COD, total dissolved solid (TDS), microbial count in BDSW were estimated as per standard methods. BDSW was characterized for Colour using UV visible double beam spectrophotometer and later calibrated against COD.

Mixed microbial consortium (MMC) was raised from Black cotton soil (BCS) on sterile minimal media (MM) broth containing Glucose (5 g/l), Na_2HPO_4 (6 g/l), NH_4Cl (2 g/l), $MgSO_4$ (0.1g/l), KH_2PO_4 (3g/l), NaCl (5g/l) and Yeast Extract (2g/l) with pH 7 at 35 ± 2^0C under shaking flaskcondition(120 rpm). Bacterial colonies from MMC (24 hour freshly grown) were counted using Quebec colony counter, followed by isolation and characterization of predominant melanoidin degrading bacterium by enrichment culture technique. Bacterial isolate was purified by repeated streaking on MM agar plate and preserved on agar minimal salt glucose at 4^0C for further use.

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2.2 Decolourisation studies:

A comparative study of decolourisation and growth was performed in four different medium A, B, C, and D to evaluate the best medium for decolourisation. Bacterial consortium & bacterium isolate was inoculated separately in sterilized 100 ml of medium and incubated at 35 ± 2^0C along with control under shaking flaskcondition (120rpm). The volume for seeding inoculums was 1 ml of unfrozen cell suspension (in log phase) developed on MM and transferred aseptically to media's mentioned in table.

Table 1: Media used for decolourisation study

Sl. No	Media	Constituents
1	*Media A*	Minimal Media (MM) broth enriched with 5% Biomethanated Distillery Spent Wash (BDSW).
2	*Media B*	Nutrient Media (NM) broth enriched with 5% BDSW containing Peptone (5g/l), Beef Extract (3g/l), NaCl (5g/l) supplemented with pH 7 and
3	*Media C:*	5% BDSW (v/v) without any supplement.
4	*Media D*	Undiluted BDSW (100% v/v) at pH 7.

Media showing highest decolourisation was selected for all the further studies. After selecting the media, the studies were carried out on different mode of operation.

2.2.1 Static-batch mode

100 ml of media supplemented with different percentage of BDSW (%v/v.) was prepared in 250 ml Erlenmeyer flask. By varying the percent of BDSW supplementation, the COD was controlled. The sterilized media was inoculated with 1ml of Bacterial culture. All the flasks were incubated at 35 ± 2^0C under static condition. After 17 days percentage of decolourisation was calculated.

2.2.2 Agitation-batch mode

In agitation condition the rotatory and mixing action increases the availability of the media constituents to the bacteria, which may increase the percentage decolourisation of BDSW. 100 ml of media was supplemented with different percentage of BDSW (v/v). The sterilized media were inoculated with 1 ml of Bacterial culture (in log phase) and kept for incubation at 35 ± 2^0C under rotary shaking at 120 rpm. During the incubation period for 24 hours, growth kinetics and percentage of decolourisation was analysed.

Based on decolourisation results, an appropriate operational mode was selected and studies on decolourisation by serial inoculation were performed to improve the overall decolourisation process.

2.2.3 Decolourization via Serial inoculation

1 ml of bacterial inoculum was inoculated into media supplemented with 10% BDSW & Incubated for 24 hours under shaking flask condition (120 rpm) at 35 ± 2^0C. Bacterial population acclimatized to 10% BDSW supplemented media was transferred into 20% BDSW supplemented media. In the same way the consortium was acclimatized step by step to the higher strength BDSW. Prior to each transfer the degradation of melanoidin was monitored in terms of decrease in the colour intensity and bacterial growth at 475 and 620 nm respectively.

2.3 Kinetics of growth & Decolourisation

2 ml of samples were collected aseptically for every two hours. The samples were centrifuged using cooling centrifugation (4^0C) at 12,000 rpm for 10 minutes to harvest the bacterial cells. The supernatant was read for OD_{475} and percentage of colour removal was

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calculated as the decrease in colour intensity of the decolourized sample against that of initial sof the original BDSW:

Percent colour removal = $\frac{Initial\ OD - Final\ OD}{Initial\ OD} \times 100$

The cell palette obtained was dispensed in equal quantity of distilled water and OD_{620} was measured against water as blank, subsequently the dry cell weight was estimated by drying the harvested cells in hot air oven (70^0C) to constant weight. The entire assay were performed in triplicates, the results interpreted are averages and compared with control.

3. RESULT AND DISCUSSION

3.1 Characteristics of BDSW & Microbial population

Anaerobically digested distillery spent wash collected after biomethanation process was dark brown in colour. BDSW showed high COD (by open reflux method) of 22,000mg/l (σ=50mg/l) and TDS of 0.225g/ml. The microbial count in BDSW showed a total of 32×10^8 CFU/ml. various dilutions of BDSW exhibited perfect positive correlation with COD, which means colour of the spent wash is responsible for high COD.

Bacterial consortium was successfully isolated from BCS on MM agar, which had a microbial count of 51×10^6 CFU/g of dry soil. Isolation of melanoidin degrading bacterium from MMC was done using enrichment culture technique. The isolate was characterized by biochemical assays, the results are mentioned in the table 2. The isolate was Gram negative rod shape. Biochemical test indicated positive (+) only for Voges prausker and Citrate test, and negative (-) for all other tests.

Table 2: Characterization of the bacterium isolate

Sl. No.	Biochemical Test	Results
1	IMViC TEST	
a)	Indole test	-
b)	Methyl red test	-
c)	Voges prausker test	+
d)	Citrate test	+
2	Starch hydrolysis	-
3	Hydrogen sulphide test	-
4	Carbohydrate fermentation	
a)	Glucose	-acid,-gas
b)	Lactose	-acid,-gas
c)	Sucrose	-acid,-gas

3.2 Decolourisation studies:

1 ml of unfrozen cell suspension of Bacterial cells developed on MM was used as seeding inoculums. The decolourizing ability of consortium and pure isolate was investigated separately in different media along with control and results are mentioned in the table 3.

Table 3: Percent decolourisation in different media

Sl. No	Media	Percent Decolourisation with consortium	Percent Decolourisation with isolate
1	*Media A*	82.36%	77%
2	*Media B*	38.55%	25%
3	*Media C:*	Growth without any	No growth with no

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		decolourisation	decolourisation
4	*Media D*	No growth with no decolourisation	No growth with no decolourisation

Bacterial isolate showed 77%, 25% decolourisation in media A & media B respectively. While in media C and media D there was no observable growth and decolourisation. Consortium decolourized the BDSW most effectively in presence of media A (82.36% reduction). Microbial growth kinetics study indicate that growth rate of bacterial consortium was more in MM compared to NM. Therefore, all the subsequent studies were performed in MM and consortium was used as a microbial source.

Agitation had a significant effect on decolourisation process. Under static-batch mode the bacterial consortium showed maximum of 80% decolourisation at 37^0C after 17 days of contact time. And under agitation-batch mode the consortium showed maximum of 82% colour removal at 37^0C after 24 Hours of contact time. Although colour removal under agitation gave best result in shorter time, but static mode is also satisfactory. If one has to make a choice between agitation mode and static mode, the best mode would be static because it gives approximately 80% decolourisation and it is saving the energy of agitation & mixing during the treatment process.

Figure 1: Biomass growth with consortium under static mode

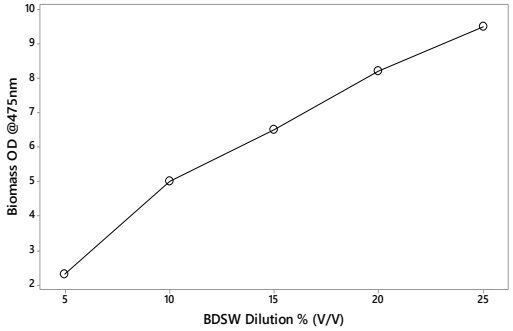
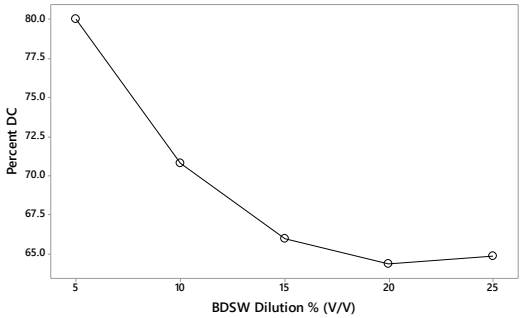


Figure 2: Percentage Decolourisation (DC) with consortium under static mode



. **Figure 3: Biomass growth with consortium under agitation mode**

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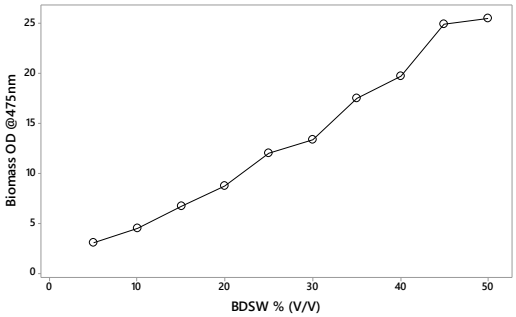


Figure 4: Percentage Decolourisation (DC) with consortium under agitation mode

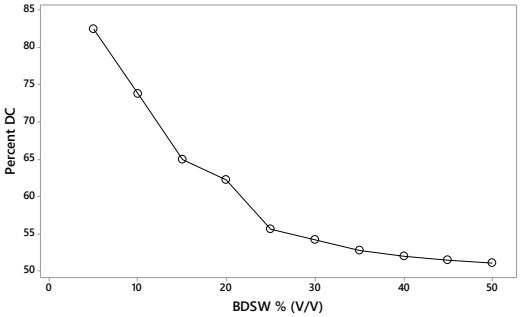


Figure 5: Percentage Decolourisation (DC) with consortium via serial inoculation

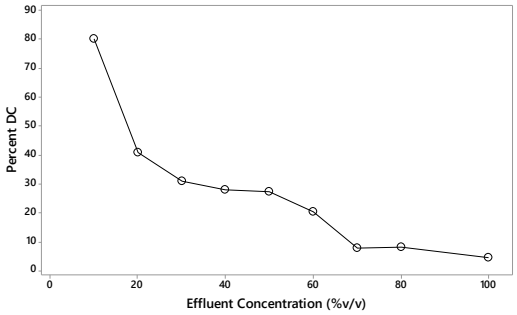
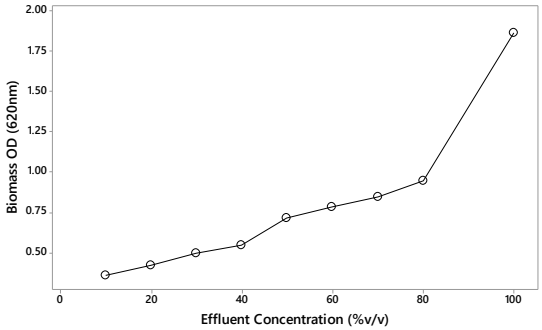


Figure 6: Biomass growth (620nm) with consortium via serial inoculation



When consortium was transferred step by step to high strength BDSW the percentage of decolourisation decreased. Same pattern was also observed when the bacterial isolate was transferred step by step to high strength BDSW, the decolourisation dropped rapidly.

4. CONCLUSION

Current investigation reports on application of bacterial consortium and bacterial isolate for biodegradation and decolourisation of biomethanated distillery spent wash. The results indicate that bacterial consortium could be a better choice over isolate for removal of coloured pollutants from distillery spent wash, since it showed the highest ability to

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decolourise the BDSW in agitation batch mode. Decolourisation using consortium supplemented with MM composition showed significant colour reduction compared to decolourisation of BDSW supplemented with NM. But when the same consortium was transferred step by step to high strength BDSW supplemented MM, the DC potential was decreased. This study concluded that the supplementary media components of MM are essential for bacterial decolourisation and degradation of BDSW.

In our investigation, decolourisation is found to be more in agitation mode, but static mode also gave comparable results. Soni Tiwari *et al.*, 2014 reports that consortium exhibited 82% decolourisation under static condition (31). Our results are line with earlier reported results. One of the major drawbacks with static mode is more contact time then agitation mode. But it can save energy on mixing during aerobic treatment. Hence, the developed bacterial consortium could be high prospect for decolourisation of melanoidin containing molasses based distillery effluent for environmental safety.

CONFLICT OF INTEREST

None

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PHYSICO-CHEMICAL CHARECTERISATION OF DISTILLERY YEAST SLUDGE (DYS) AND UTILIZING IT FOR ENHANCING SOIL QUALITY

Supriya S Doijode[1]*, Sreedhar Reddy[2], Ishwar Chandra[3]

[1]Research scholar, Department of Biotechnology, REVA university, Bangalore.

[2]Department of Biotechnology, REVA university, /Bangalore.

[3]Department of Biotechnology, M Visvesvaraya Institute of Technology, Krishnadeveraya Nagar, Hunasamaranahalli Bangalore-562157

ABSTRACT: Distillery yeast sludge (DYS) refers to yeast retained at the bottom of fermentor tank in the form of thick sludge during the alcoholic fermentation process in molasses based distillery industries. It is sometime called as spent yeast or trub. It is mostly left as waste product which is donated to nearby farmers to be used as poultry feed. Distillery industry produces huge quantity of DYS which is difficult to manage and dispose, which later leads to environmental problems. The following study was, therefore, planned to utilize this DYS for better use. In order to do so protein concentration, vitamin (water soluble and fat soluble vitamins) concentration, free amino acid concentration was determined by standard methods. DYS was found to contain very high amount of Niacin (224.12 µg/100g), pyridoxine (158.61 µg/100g), Pantothenic acid (594.62 µg/100g). It also consist of very high proportion of Vitamin E (1677.83 µg/100g), free amino acid (51.56 mg/g) and protein concentration (39.98mg/g). After blending the soil with DYS and incubating for 45 days , the NPK levels of soil was increased significantly.

KEYWORDS: *Distillery yeast sludge, Vitamin E, Pantothenic acid, Amino acid, Protein*

***Corresponding Author: Supriya S Doijode**

Research scholar, Department of Biotechnology, REVA university, Bangalore.
Email Address: supriya891@gmail.com

1. INTRODUCTION

Molasses the waste materials from the sugar industry is used as the substrate for the production of ethanol by yeast, mainly S.cerevisiae (1). Distillery yeast sludge is a solid waste product generated in the molasses based distillery industry. After filtration of fermented broth huge amount of yeast sludge is left as waste. According to latest reports, there are around 397 operating distilleries in India (2)(3). Yeast sludge, a by-product of molasses based industries, has an availability of one million ton per annum (4). Disposal of huge volume of yeast sludge is a big problem. Yeast has been used as feed and fodder successfully for certain animals such as horses, cows, and poultry. It contains a high percentage of protein (21%) and essential amino acids such as lysine, methionine, glycine, arginine, leucine, and histidine (5). The feasibility of vermicomposting technology to stabilize the distillery industry sludge mixed with a bulking agent (cow dung) in different proportions has been studied by some authors (6).

Several studies have been reported to utilize different species of yeasts and molds along with agro-industrial wastes for single cell protein production. Some authors have also reported the utilization of yeast sludge as a source of Single cell proteins (SCP).

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Microorganisms are great sources SCP of due to their high efficiency of converting carbon source to protein, rapid growth rate and ability to synthesize inexpensive substrates as carbon sources (7). SCP are microbial dried cell mass of algae, fungi and bacteria, which contain high concentration of nucleotides, inositol, and glutamic acid. It can also contain other biological molecules like lipids, carbohydrates, and vitamins. And they are known as bioprotein, or microbial protein (8)

Hence, the study has been carried out to probe the nutritional analysis of dried distillery yeast sludge. The results would provide baseline information that would lead to evaluate the potential for production of different value added products

2. MATERIALS AND METHODS

4.1 Collection of Distillery yeast sludge

Distillery yeast sludge was collected from one of the reputed industry in Karnataka. Sludge was collected in a clean sterilized polythene bag. Sludge was dried in shade at room temperature, for 15 days. Dried yeast sludge was tested for moisture content and stored at room temperature for further use.

2.1 Nutritional analysis:

Distillery yeast sludge was tested for following nutritional content by standard methods.

2.1.1 Protein analysis

1g of sample was thoroughly grind with distilled water. The sample was centrifuge (extract) for 10 min. at 10000 rpm. the supernatant was collected and made up the volume to 25 ml. 0.1 ml of supernatant was taken in clean test tubes and made up the volume to 1 ml by adding distilled water. 5 ml of reagent X (Mix equal volume of reagent 2 and reagent 3. Take 0.2 ml and 9.8 ml of reagent 1 (For 10 ml). Prepare fresh solution on the day of work) was added. Thorough mixing was done and it was allowed for standing at RT for 10 min. later 0.5 ml of Folin-Ciocalteau's reagent (1:1) was. Incubation of all the tubes was done for 30 min at dark. Intensity of color developed was read by measuring the absorbance at 750 nm.

Calculation:

$$\text{Proteins (mg/g)} = \frac{OD_{750nm} \times \text{Std. value (µg/OD)} \times \text{Total Vol. of extract}}{\text{Assay volume} \times \text{wt. of tissue (g)}} \times 1000$$

2.1.2 Amino acid analysis

Two gram of sample was homogenized in 10 mL of 80% ethanol later centrifuge, supernatant was collected and volume was made upto 25 mL. Pipette 5 mL of supernatant was evaporated to dryness than residue was dissolved in 20 mL distilled water. Then, 1 mL of extract and 3 mL water was transferred in test tubes later 1 mL of ninhydrin reagent was added to all the test tubes including the test tubes labelled blank and unknown. Contents were mixed the and all the test tubes were placed in boiling water bath for 15 minutes. Test tubes were cooled under running tap water and 1 mL of ethanol was added to each test tube and mixed well. absorbance at 570 nm was recorded.

Calculation:

$$\text{Free amino acids (mg/g)} = \frac{OD570nm \times \text{Std. value (µg/OD)} \times \text{Total Vol. of extract}}{\text{Assay volume} \times \text{wt. of tissue (g)} \times 1000}$$

2.2 Vitamin analysis

2.2.1 Extraction of water soluble vitamins

Water soluble and fat soluble vitamins extraction procedure is followed as described by

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Santos *et al*. (2012). Two gram of sample is extracted with 20 mL of 10 mM ammonium acetate: methanol 50:50 (v/v) containing 0.1% BHT. After homogenization, samples were placed in the shaker water bath at 70ºC for 40 minutes. Centrifugation of the sample was done at 10000g for 15 min and the supernatant was collected and made up to the volume to 25 ml (residue to be kept fur further analysis). Supernatant was filter through 0.2µm nylon filter membrane and injected 5µL to UPLC-MS/MS system to determine the water soluble vitamin content. The analytical column 2.1 X 50 mm UPLC BEH-C18 is used for the water soluble and fat soluble vitamins analysis. During the extraction process, samples were always protected from direct exposition to light and kept on ice bath to minimize vitamins degradation.

Mobile Phase

Solvent - A: 0.1% formic acid in water

Solvent - B: Acetonitrile

2.2.2 Extraction of fat soluble vitamins

The solid residues from the water-soluble vitamins were re-extract twice with 25 mL each ethyl acetate. Centrifugation of the sample at 10000g for 15 min was done. Supernatant was taken and evaporated completely to dryness using flash evaporator. the residue was dissolved in 1 mL of mobile phase and filter through 0.2 µm nylon filter membrane and inject into 6µL to UPLC-MS/MS system to monitor the fat soluble vitamin contents.

Mobile Phase

Solvent - A: Acetonitrile

Solvent - B: 0.2% formic acid in methanol

2.3 Production of value added products

Soil was amended with distillery yeast sludge to check any enhancement in nutritional quality of the soil. table 1 represents diffrent combinations soil and distillery yeast sludge tried. The soil samples were stored in room temperature for 45 days. After incubation time was over the samples were analysed for the nutritional content mainly Nitrogen Phosphorous and potassium (NPK) levels. The entire test was performed in duplicate and the results shown are average.

Table 1: Different combinations of soil and DYS

Sl. No	Samples	Combinations
1	Tube 1	2gm sludge + 8gm soil
2	Tube2	5gm sludge + 5gm soil
3	Tube3	1gm sludge + 9gm soil
4	Tube4	8gm sludge + 2gm soil
5	Tube 5	Only sludge

3. RESULTS AND DISCUSSION

Table 2: Values of nutritional content in DYS

Sl. No.	Component	Nutritional analysis	
1	Protein:	39.98mg/gm	
2	Free amino acids:	51.56mg/GMC	
3	Vitamins	**Water soluble**	**Value**
		Thiamine	43.60 ug/100gm
		Niacine	224.12 ug/100gm

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		Pyridoxine	158.61 ug/100gm
		Panthothenic	594.62 ug/100gm
		Biotin	3.17 ug/100gm
		Riboflavin	3.04 ug/100gm
		Folic acid	0.22 ug/100gm
4	Vitamins Fat soluble	Vitamin K2	0.30ug/100g
		VitaminD2	7.15ug/100g
		Vitamin D1	3.43ug/100g
		VitaminE	1677.83ug/100g
		Vitamin K1	3.52ug/100g

Protein content is extremely rich 39.98mg/gms, hence can be used as a very good source of Proteins.

Free amino acid is used for a variety of applications in industry, but their main use is as additives to animal feed. This is necessary, since many of the bulk components of these feeds. The food industry is also a major consumer of amino acids. The chelating ability of amino acids has been used in fertilizers for agriculture to facilitate the delivery of minerals to plants in order to correct mineral deficiencies, such as iron chlorosis. These fertilizers are also used to prevent deficiencies from occurring and improving the overall health of the plants. Similarly, some amino acids derivatives are used in pharmaceutical industry as well. And sludge contains 51.56 mg/gms of amino acids.Water soluble vitamins are also present in yeast sludge as mentioned in the table 1 above. Thiamine is 43.60 ug/100gms, Niacine is 224.12 ug/100gms,Pyridoxine is 158.61 ug/100gm ,Pantothenic is highest 594.62 ug/100gms,biotin is 3.17ug/100gms,Riboflavin is 3.04 ug/100gms and Folic acid is 0.22ug/100gm.

Vitamin fat soluble are also present and the concentration of Vitamin K2 is 0.30ug/100g, Vitamin D2 is 7.15ug/100g, Vitamin D1 is 3.43ug/100g,vitamin E is highest 1677.83 ug/100gm content and Vitamin K1 is 3.52 ug/100gms.

Table 3: NPK levels in soil amended with DYS

Sl. No.	Samples	Nitrogen	Phosphorous	Potassium
1	Tube1	1.37	0.36	1.88
2	Tube2:	1.51	0.29	2.04
3	Tube3:	1.22	0.14	1.76
4	Tube4:	1.60	0.27	2.32
5	Tube5	1.44	0.21	1.54

After the incubation of the tubes for 45 days and later testing the concentration of the soil, we can come to a conclusion stating yeast sludge acts as very good source of NPK and enrich the nutrient content in soil , Tube no. 4 has 8 gms of sludge and 2 gms of soil and the concentration of Nitrogen is 1.60 mg/gms, phosphorus is 0.27 mg/gms and potassium is 2.32 mg/gms.

4. CONCLUSION

Distillery yeast sludge which is difficult to manage and dispose bears high commercial potential. DSY can be employed for the production of biofertilizer by amending with soil, DYS has high strength in increasing the NPK levels of the soil, after blending the it with DYS and incubating for 45 days , thereby increasing the quality of the soil. DYS was found to contain very high amount of Niacin (224.12 µg/100g), pyridoxine (158.61 µg/100g), Pantothenic acid (594.62 µg/100g). It's a rich source of Vitamin E (1677.83 µg/100g), free amino acid (51.56 mg/g) and protein concentration (39.98mg/g).

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CONFLICT OF INTEREST

None

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STUDY OF ANTI-INFLAMMATORY EFFECT OF *TERMINALIA ARJUNA*

Jayashree S[1] Chaithra C[1] and Ranjitha S B[2]
[1]Department of Biochemistry, REVA University, Kattegenahalli, Bangalore 560064.
[1]Department of Biochemistry, REVA Institute of Science and Management, Bangalore.

ABSTRACT: *Terminalia arjuna* has been widely used in Ayurvedic medicine for the treatment of Cancer, Blood pressure, Diabetics, Ulcers and Heart diseases. The present investigation, was aimed to evaluate the anti- inflammatory activity of *Terminalia arjuna*. Initially, the bark extract in methanol had shown the presence of phytochemicals *Terminalia* leaf contains 63 types of flavonoids out of which 27 are predominant. The extract has shown proliferative effectiveness on hydroxyl free radical scavenging assay. The flavonoids are of great importance as antioxidants. Flavonoids have the capacity to quench free radical (hydroxyl radical) and may show remarkable antioxidant activity. The *Terminalia arjuna* extract was evaluated for presence of Quercetin, by High Performance Liquid Chromatography(HPLC). The HPLC analysis of *Terminalia arjuna* extract showed the presence of quercetin.(1.07 µg/g of extract) Quercetin is said to be one of the most widely used bioflavonoids for the treatment of metabolic and inflammatory disorders. Quercetin inhibits inflammatory enzymes cyclooxygenase (COX) and lipoxygenase thereby decreasing inflammatory mediators such as prostaglandins and leukotrienes.

Thus the present investigation revealed that *Terminalia arjuna* has shown promising anti-inflammatory and anti- oxidant activity.

KEYWORDS:: Anti-inflammatory, Hydroxyl radical scavenging assay, Phytochemicals.

Corresponding Author: Dr. Jayashree S
Department of Biochemistry, REVA university, Kattegenahalli, Bangalore, Karnataka
Email Address: jayashrees@reva.edu.in

1. INTRODUCTION

Terminalia arjuna is a tree belonging to the family Combretacea**, (**Roxb Wight Arn) and originated from India whose bark has been used in medicine for over three centuries in India.[1] Since it contains phenols, flavonoids, tannin, saponins, alkaloids, glycosides, phytosterols and carbohydrates,[2] studies reveal that these compounds act as antioxidants by inhibiting oxidative mechanism caused by reactive oxygen radicals such as hydroxyl, peroxide and hydroperoxide[3] and protect against diseases such as inflammatory diseases, cancer, coronary heart disease and Alzheimer's disease [4-9]. An unrestrained and persistent inflammation may act as an aetiological factor for many of these diseases.[10]. The above mentioned compounds also act as mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases. The bark extract has a significant prophylactic heart against catecholamine induced Congestive Heart Failure (CHF) and antioxidant enzymes activities.[11] Antioxidant compounds terminate the chain reactions by removing free radicals intermediates and inhibit other oxidation reaction by oxidization.[12]. *Terminalia arjuna* bark extract exhibits the antidiabetic activity by enhancing the peripheral utilization of glucose by correcting the impaired liver and kidney glycolysis by limiting its gluconeogenic formation(insulin)[13].

Quercetin is an important flavonoid known to possess an enormous array of pharmacological activities and it regulates the repression of miRNA in inflammatory response.[14] The

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present investigation shows the presence of quercetin and hydroxyl radical scavenging activity to prove for its anti-inflammatory properties and can be explored for its highest therapeutic efficacy to develop safe drugs for various inflammatory disorders.

Our studies focuses on investigation of anti inflammatory and antioxidant properties of *Terminalia arjuna* through selected compound(Quercetin) involved in inflammatory disorders and radical(Hydroxyl) scavenging assay.

2. MATERIALS AND METHODS

The bark samples of *Terminalia arjuna* were collected and authenticated from the Amurth Kesari department, GKVK Bangalore.

2.1 Preparation of extract: Samples were air dried at room temperature and homogenised to a fine powder and stored in the bottle for experimental studies.

The shade dried and coarsely powered stem bark (1kg) was extracted with 50% ethanol (1.5L) in the cold condition for 72 hours according to the traditional system of medicine. The extract was filtered and distilled, a reddish brown syrupy mass was obtained. It was finally dried at low temperature under reduced pressure in a rotary evaporator. A crude residue (75g) was obtained giving a yield of 7.5%. When needed, the crude extract was suspended in distilled water and used in the study for the following analysis.

2.2 Phytochemical analysis

The preliminary phytochemical screening of extracts of *Terminalia arjuna* was carried out according to the standard methods. The phytochemicals such as flavonoids, phenols, tannins, alkaloids, terpenoids, reducing sugar, saponins, quinines, proteins and steroids was evaluated, by using standard protocols.[15]

2.3 Hydroxyl radical scavenging assay

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The extract on the inhibition of free radical was assessed by the method of deoxyribose.[16]

2.4 HPLC analysis

10grams of plant bark powder was extracted with 50ml of methanol at 50^{o} C for 4hours. The methanoic extracts were filtered through Whatman No 1filter paper and filtrate was evaporated to dryness. Methanoic extract (10mg/ml) was used for HPLC analysis. Standard and the isolated fraction of quercetin were analysed by HPLC technique with the optimized chromatographic conditions by using HiQ Sil C18HS column with the size carrying the dimension of 4.6mmX250mmX5µ. Methanol:0.1% Ortho phosphoric Acid (65:35%) are used as a mobile phase with the flow rate as 1ml/min. The injection loop capacity is of 10µl along with retention time of 8.4min with running period of 13minutes. The concentration of the sample was taken for 10mg/ml along with standard Quercetin by using UV detector at wave length of 369nm and was quantified.[17]

3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis (Qualitative) of *Terminalia arjuna* bark extract

The Qualitative analysis shows the presence of phytochemicals in the extract which are mentioned in the table 1

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Table-1

SI no	Tests	Observation
1	Alkaloids	+
2	Terpenoids	+
3	Phenol and tannins	+
4	Reducing sugar	+
5	Saponins	+
6	Flavonoids	+
7	Quinines	+
8	Protein	+
9	Steroids	+

'+'presence : '-'Absence

3.2 Hydroxyl radical scavenging assay

The extract possess concentration dependent activity on hydroxyl radical scavenging assay and are illustrated in the **Fig 1** and **Fig 2.***T.arjuna* showed IC50 values of **30.35µg/ml** as compared with standard (quercetin) showed IC50 value of **28.01µg/ml**. Thus the scavenging activity of plant extract was mild at lower concentration of extract and gets increased as the concentration of extract increased and showed good activity as compared with standard quercetin. Plants such as *P.granatum, A.heterophyllum* and *Bacopa monnieri Linn* showed the hydroxyl radical scavenging action.*P.granatum* fruit shows the anti inflammatory activity through the carrageenan, dextron, BSA and formalin induced by acute and chronic inflammation. [18] *A.heterophyllum* shows anti-inflammatory by inhibiting prostaglandin pathway.[19] Similarly *Bacopa monnieri Linn* significantly inhibits lipoxygenase and cyclooxygenase activities exhibiting the anti inflammatory activity.[20]

Thus the extract chosen for the study holds scavenging property of free radicals through their antioxidants content and earlier investigations proven its potency towards health proliferative effectiveness.

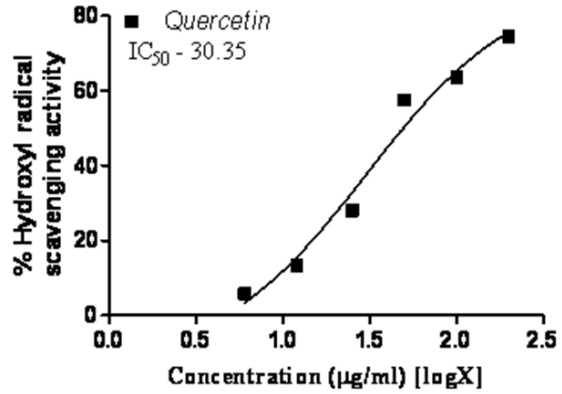

Fig 1: Hydroxyl radical scavenging activity of Quercetin(Standard0.

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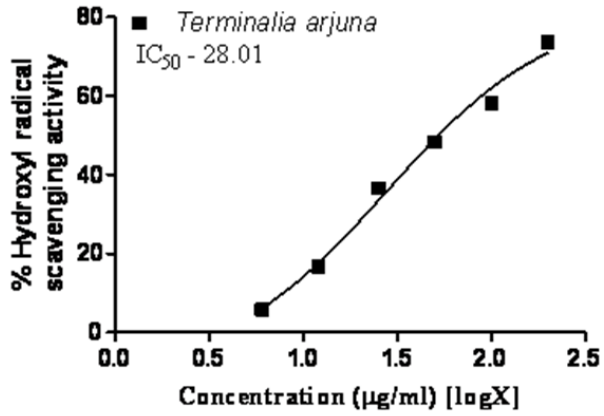
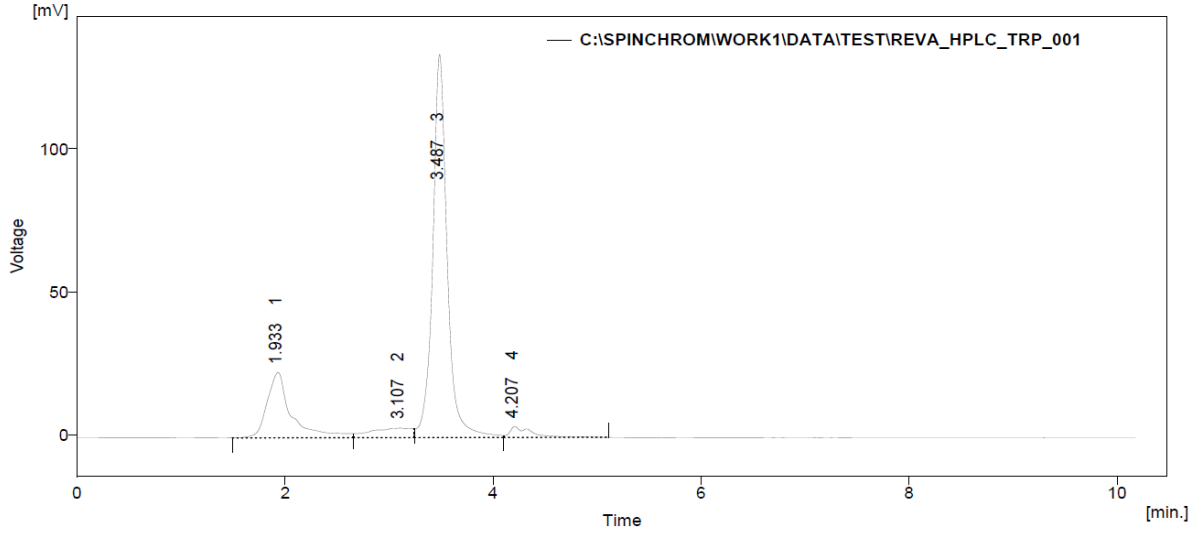


Fig2: Hydroxyl radical scavenging activity of *Terminalia arjuna*

3.3 HPLC analysis of quercetin

The *Terminalia arjuna* extract was evaluated for presence of Quercetin, one of the vital flavonoids present in plant by HPLC. The Quercetin standard eluted at retention time of 3.48 minutes **(Fig. 3)**. The HPLC analysis of *Terminalia arjuna* extract presented the presence of Quercetin **(Fig. 4)**. The area of standard Quercetin was compared with that of extract and content(Quercetin) was estimated to be **1.07** ug/g of Quercetin in the extract.



Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\TEST\REVA_HPLC_TRP_001)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

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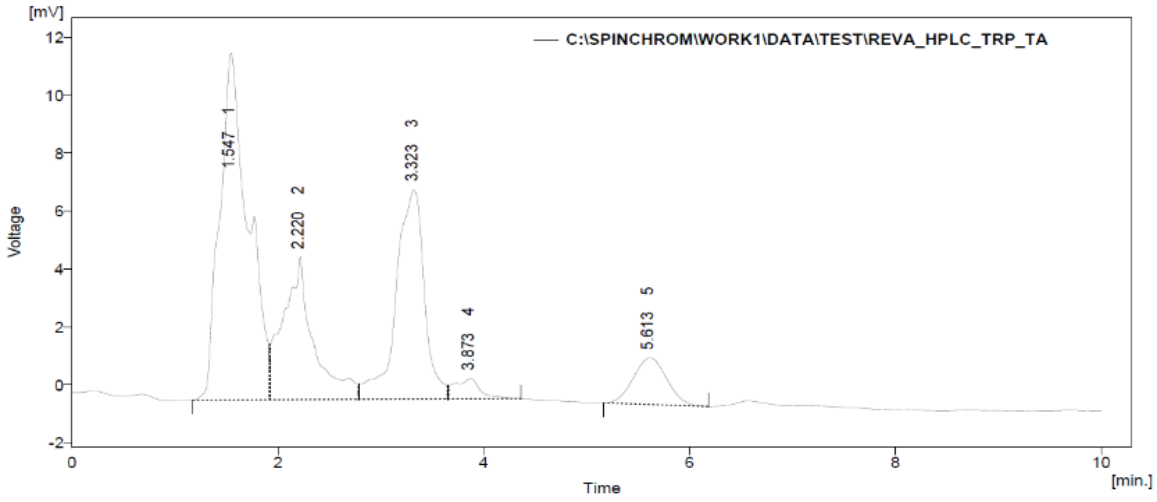
Fig 3: HPLC graph for standard(Quercetin)



Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\TEST\REVA_HPLC_TRP_TA)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.547	229.640	11.970	44.3	45.3	0.27
2	2.220	101.600	4.922	19.6	18.6	0.28
3	3.323	138.804	7.220	26.8	27.3	0.28
4	3.873	12.358	0.706	2.4	2.7	0.30
5	5.613	36.406	1.612	7.0	6.1	0.36
	Total	518.808	26.429	100.0	100.0	

Fig 4: HPLC graph for plant Sample(*Terminalia Arjuna)*

Quercetin can protect against environmental causes of free radicals such as smoking. Cigarette tar is a source of free radicals which has been found to damage erythrocyte membrane. It is also found that the Quercetin and its metabolities could protect erythrocyte from the membrane damage that is caused by smoking.[21] Quercetin has significant role on acute and chronic inflammation[22] Quercetin fight against the inflammation and prevents the cardiovascular diseases.[23] Quercetin inhibits *invitro* production of cyclooxygenase and lipoxygenase which are typically induced by inflammation.[24] Quercetin has an inhibitory effect on the proliferate effectiveness on the cellular migration and on the accumulation of the collagen in the injured sites. Quercetin is an eosinophilic inflammation suppressor [25] and exhibit important vaso relaxant properties on isolated arteries which helps to lower blood pressure and prevents the development of cardiac hypertrophy.[26] Quercetin normalizes the blood glucose levels by increasing the intracellular transport of glucose and by improving insulin.[27] Quercetin involve in the stimulation of of glucose uptake through an Mitogen-activated protein kinase(MAPK) insulin-dependent mechanism by translocation of glucose transporter 4 (GLUT4).[28]

4. CONCLUSION

Thus our present investigations revealed the methanol extract of *Terminalia arjuna* has good scavenging action on hydroxyl radicals in a dose dependent manner**.** Additionally HPLC

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analysis showed the presence of Quercetin. Both the quantifications displayed the promising anti-inflammatory activity since the previous findings of other plant extracts correlates antioxidant and anti-inflammatory properties which are involved in therapy of various diseases including diabetic complications, cardiovascular diseases concerned with inflammation. Further research on the underlying mechanism of *Terminalia arjuna* inducing cell proliferation and the identification and isolation of pure compounds will be necessary to determine the cellular targets.

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INFLUENCE OF *CYPERUS ROTUNDUS* ON DIABETIC COMPLICATIONS THROUGH *INVITRO* STUDIES

Jayashree S[1], Shalini S[1], Likitha PM[1]
Department of Biochemistry, REVA University, Bangalore

ABSTRACT: *Diabetes mellitus* (DM) is a group of metabolic disorders in which there are high blood sugar levels over a prolonged period and its complications are retinopathy, neuropathy and nephropathy. The major chemical components of the selected plant tuber *Cyperus rotundus* posssesses various pharmacology activities to cure various disorders such as diuresis, inflammation, dysentery ,rheumatism and diabetics. One of the component Gallic acid in the selected herb was quantified by High Performance Liquid Chromatography(HPLC).Gallic acid possess diverse biological and pharmacological activities through radical scavenging, interfering with the cell signaling pathways, apoptosis of various diseased cells and *In vivo* and *in silico*studies. Blood samples of diabetic patients were collected to analyse biochemical parameters related to diabetic complications such as glucose level, lipid profile, glycosylated haemoglobin(hbA1c) in the blood samples by subjecting them with the extract. The positive results clearly established the potency of *C.rotundus* on diabetic complications.

KEYWORDS: Diabetes mellitus, diabetic complications, HPLC, gallic acid**,** *Cyperus rotundus.*

Corresponding Author: Dr Jayashree S * Ph.D.

Department of Studies in Biochemistry, REVA University, Yelahanka, Bengaluru-64, India.
Email Address:jayashrees@reva.edu.in

1. INTRODUCTION

Diabetes mellitus (DM) a group of metabolic disorders where the high blood sugar levels are observed for a long period. Symptoms of high blood sugar include frequent urination, increased thirst and hunger[1]Type I DM called as Insulin-dependent diabetes mellitus "(IDDM) or "juvenile diabetes" and Type II DM as "non insulin-dependent diabetes mellitus "(NIDDM) or "adult-onset diabetes". Complication of Diabetes mellitus are Diabetic retinopathy, neuropathy and nephropathy. The primary strategy for avoiding complications is prevention[2]Blood glucose control has been shown in major clinical trials to reduce the risk of microvascular complications, and is now also thought to stave off macrovascular complications. [3]

There are various approaches to reduce the ill effects of diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. A list of medicinal plants with proven antidiabetic and related beneficial effects and of herbal drugs used in treatment of diabetes is compiled. These include, *Allium sativum*, *Eugenia jambolana*, *Momordica charantia Ocimum sanctum*, *Phyllanthus amarus*, *Cyperus rotundus*, *Trigonella foenum graecum* and *Withania somnifera*. The plant chosen for the study is *Cyperus rotundus*. [4]*Cyperus rotundus* is a species of sedge(Cyperaceae) native of Africa, southern and central Europe and southern Asia.[5] . *C. rotundus* has many beneficial uses. It is a staple carbohydrate in tropical regions for recent hunter-gatherers and is a famine food in some agrarian cultures [6]The major chemical components of this herb are phenols, flavonoids, terpenoids, cyperol,cyprotene, cadalene acyerone,etc. Earlier research studies have shown that it posssesses various pharmacology activities such as anti- inflammatory, anti-dysenteric

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,anti-diabetic activities etc. [7]

Our investigations were focused with identification of chemical component in the selected plant tuber Gallic acid(a phenolic compound) andits therapeutic influence on diabetic complications through *invitro* assays carried out in blood samples.

2. MATERIALS AND METHODS

2.1 Preparation of the plant extract (*Cyperus rotundus)*

C.rotundus tubers were collected from local market, Bangalore and were cleaned.They were authenticated by Dr. Jawahar, Botanist, Institute of Ayurveda & Integrative medicine (FRLHT) Bangalore and Authentication Number: FRL-CPP-EST-015-579.

The tubers were then powdered and sieved .The finely powdered tubers were weighed (40g) to which ethanol(500ml) is added and then subjected to filtration and rotatory evacuator. The obtained plant extract is taken for analysis such as (i) Quantification of Gallic acid(HPLC) and (ii) *Invitro* assay.

2.2 Quantification of gallic acid (HPLC)

Gallic acid content in the sample(10µl injected) was quantified by HPLC using the method ofMahfuzElmastas *et al*.,2003[8]using standard concentration as 100ug/ml with methanol. Shimadzhu LC- Prominence 20AT Instrument was used with C18 column holding dimension of 250 mm x 4.6 mm with 5u particle.LinearMobile Phase containing Acetonitrile (60%) and water (40%) along with Running Timeof 12min and Flow Rate as 1.0ml/min.[9]

2.3 *Invitro* assay

Invitro studies are performed using theblood samples of diabetic patients, where the biochemical parameters mentioned below are tested

2.4 Collection of blood samples

Fifteen diabetic patients blood samples of men and women were collected from Hosmat hospital with patient's concern and were grouped in five with triplicatesfor analysis.[10][11]

2.5 Isolation of erythrocytes and analysis.

20ml of blood was collected from diabetic patients in tubes containing 20mg of EDTA to prevent coagulation and centrifuged at 1000rpm for 10minutes at 4ºC.Plasma was removed carefully and the white puffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4. Washed erythrocytes were stored at 4ºC and used within 6hours.[12]50µl of diluted (100µl erythrocytes suspension: 900µl 1XPBS) erythrocytes suspensions with 100µl of test samples of different concentrationswere considered.Reaction mixture was incubated at 37ºC in a water bath for 60minutes.The volume of reaction mixture was adjusted to 1ml by adding 750µl of 1X PBS and centrifuge at 3000rpm for 3minutes and the resulting haemoglobin supernatant was measured and used for the following biochemical parameters.

1. Glucose level was assessed by the method of Zlatkis et al,1953 [13]
2. HbA1c (Haemoglobin A1c) was performed by the method of Olatunde Farombi, E et al.,2002.
3. Lipid profile test was done by the method of MahfuzElmastas et al.,2003
   - ❖ Total cholesterol
   - ❖ Serum HDL
   - ❖ Serum LDL
   - ❖ Triglycerides
   - ❖

3. RESULTS AND DISCUSSION

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3.1 Quantifiaction of Gallic acid by HPLC

The amount of gallic acid determined through HPLC of the given plant extract(10mg) was **4.36×10^{-2}mg (43.6µg)** and are represented in the fig 1.

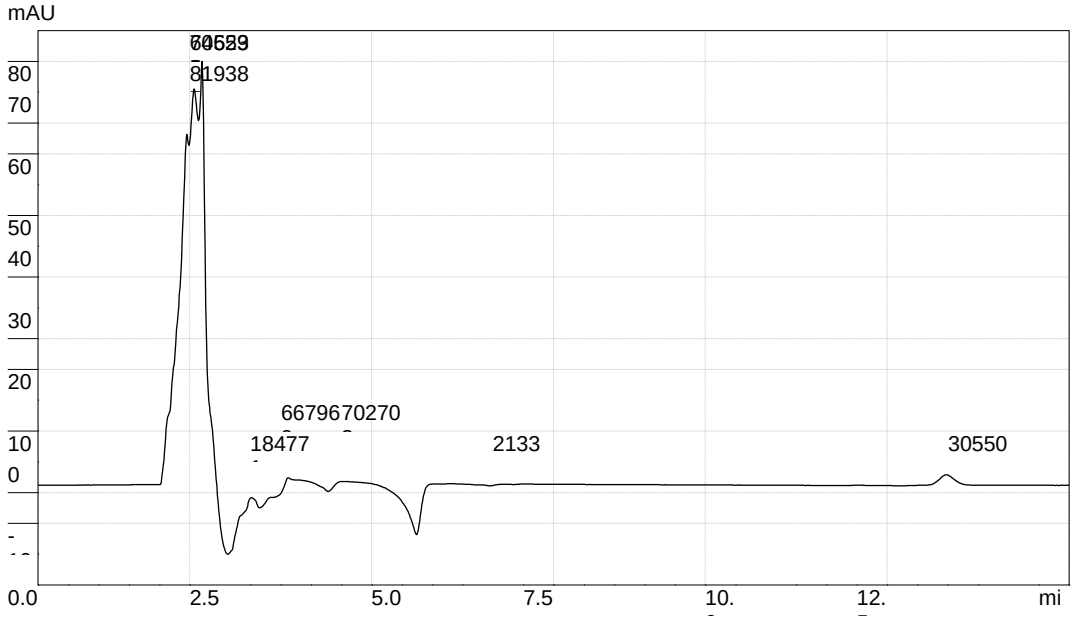

Fig1: HPLC analysis of *C rotundus*

Gallic acid (GA) (3, 4, 5-trihydroxybenzoic acid)has a phenolic groups that are a source of readily available hydrogen atoms so that radicals produced can be delocalized over the phenolic structure and thus act as a radical scavengerspossessing several pharmacological properties such as anti-inflammatory, antioxidant, anticancer, anti-diabetic etc which were shown in previous reports.Hence gallic acid could be considered as a promising lead compound for new drug development. [14]

3.2 *Invitro* assay

3.2.1 Biochemical parameters

The biochemical parameters illustrated in the table 1 and 2showed the significant effect of the extract on blood samples of diabetic subjects.The parameters such asblood glucose, lipid profile:Serum cholesterol,HDL,LDL Triglycerides andGlycosylated Haemoglobin (HbA1c) levels, showed significant decrease by the plant extract.

Table 1: blood glucose

Sl.no	1	2	3	4	5
Blood glucose level(mg/dl) Before treating with the extract	245	214.5	180	154.5	188
Blood glucose level(mg/dl) After treating with the extract	216.5	189	120	152	130.5
HbA1C(<6.0%) Before adding the extract	6.5	8.8	11.1	11.9	13.6

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HbA1C(<6.0%) After adding the extract	6	7.6	9.5	9.8	12

Values expressed as Mean ±SD for 3 subjects in each group

Table 2:lipid profile

Sl.no	1	2	3	4	5
Total cholesterol (mg/dl) Before adding the extract	110.5	182	161	185	252
Total cholesterol (mg/dl) After adding the extract	103	176	150.5	180.5	225
HDL(mg/dl) Before adding the extract	54	49	54.5	49.5	52
HDL(mg/dl) After adding the extract	36	44	44.5	41	45.5
LDL(mg/dl) Before adding the extract	79	69.5	120	82	72
LDL(mg/dl) After adding the extract	77	53	92	70	62
TG(mg/dl) Before adding the extract	322	157	149.5	121	359.5
TG(mg/dl) After adding the extract	320	125	130	112.5	321

Values expressed as Mean ±SD for 3 subjects in each group

Diabetes Mellitus (DM) is characterized by chronic hyperglycemia with disturbances of Carbohydrate, Lipid and Protein metabolism resulting from defects in insulin secretion, insulin action or both[14]Glycated hemoglobin (HbA1C) is a routinely used marker for long-term glycemic control. The importance of glycemic control in order to manage dyslipidemia and risk for cardiovascular disorder in type 2 diabetes. Elevated HbA1c has been regarded as an independent risk factor for coronary heart disease (CHD) and stroke in subjects with diabetes [16][17]. Diabetes leads to a condition called diabetic dyslipidemia, coupled with high cholesterol and high blood sugar levels having a detrimental effect on diabetes control and overall health.Hence all the related parameters show significant decrease by the influence of extract and thereby proving the phytochemicals (Gallic acid) in the tuber

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prevents diabetic complications.[18][19]

4. CONCLUSION

Significant decrease in the glucose level, lipid profile and HbA1C were observed when treated with the plant extract in the diabetic subject's blood samples. Thus it is concluded that the extract can counteract various diabetic complications through the presence of bio active compound-gallic acid holding various therapeutic properties such as anti-inflammatory, anti-diabetic , anti-oxidant etc. [20][21]Due to these activities gallic acid could be considered as a promising lead compound for new drug development.Future work is an attempt to compile literature reporting on isolation, quantification and pharmacological activities of gallic acid and other phytocompounds to provide quick access to research scholars for their research exploration.

CONFLICT OF INTEREST

None

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3D STRUCTURE PREDICTION AND IDENTIFICATION OF SMART DOMAIN SEQUENCES OF CAPRIPOX VIRUS P32 ENVELOP PROTEIN IS THE HOMOLOG OF THE VACCINIA VIRUS H3L GENE: AN *INSILICO* APPROACH.

[*1]Kiran Kumar Burranboina, [*1,3]Kumar KM, [2]Manjunatha Reddy G B, [2]Yogisharadhya R, [3]Srijeeta Nayek, [3]Rikumoni Chakroborty and [3]Snehasish Kundu.
[1]Department of Biotechnology, REVA University, Bengaluru, India.
[2]National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India
[3]Dayananda Sagar college of engineering, Bengaluru, India.

ABSTRACT: Capri poxviruses is the genus comprises Sheep pox (SPPV) and goat pox (GPV) and Lumpy Skin Disease Virus (LSDV) are OIE notifiable and economically important Transboundry Animal diseases of sheep, goats and cattle respectively. These are infectious diseases of small ruminants and were affecting agricultural economy and livelihood of the farmers in endemic regions. These diseases are serologically indistinguishable and earlier thought to be caused by a single species of virus. P32 is the one of the major immunogenic protein corresponds to an envelope protein homologous to P35 protein encoded by Vaccinia virus H3L gene, and locates on the membrane surface of the viral particle. The selected sequences were compared with other P32 sequences of capripoxviruses available in the database. The sequences were shown a major difference between them is the presence of an additional aspartic acid at 54th position of P32 of sheep poxvirus that is absent in both goat poxvirus and lumpy skin disease virus. Further, seven unique aminoacid substitutions were observed at positions 26, 46, 132, 134, 290, 305 and 322 in the sequence of sheep poxvirus, which can be taken as SPV signature residues. Sequence analysis revealed that sheep pox and goat poxviruses share 97.5 and 94.7% homology at nucleotide and amino acid level, respectively. Three dimensional structure of capripox virus major envelop protein p32 Prediction and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

Key words: Capripox viruses, Animal diseases, Vaccinia Virus, World Organization for Animal Health (OIE), and Simple Modular Architecture Research Tool.

Corresponding Auther: Kirankumar Burranboina, REVA University, Bangalore-560064. Email: kirankumarq@gmail.com. Mob: 07892693246.

1. INTRODUCTION

Capripoxvirus are large (170–260 nm by 300-450 nm), double stranded DNA, and enveloped viruses and these are the member of the Poxviridae family, characterized clinical symptoms by fever, generalised papules, vesicles (rarely), internal lesions and death. It causes diseases in sheep, goat and cattle [1] [2][3] .The Capripox viruses are among the most serious of all animal poxviruses. These genus consist of three species: the type species: sheep pox virus (SPPV), goat pox virus (GTPV), and lumpy skin disease virus (LSDV), these causes sheep pox, goat pox, and with lumpy skin disease respectively. these Capri- poxviruses of animals, important and listed in group A diseases of The World Organization for Animal Health (OIE) [4]. These viruses cause systemic disease in all ages of sheep and goats but are most severe in the young. In affected animal the mortality reaches to 100% and the affected animal shows multifocal necrotic lesion on the skin and internal organs including lungs, liver and gastrointestinal track. Diagnosis of the disease by PCR, virus neutralisation and virus

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isolation. Various strain of capripoxvirus are responsible for infection which is antigenically and serologically similar but they are distant from each other genetically.The three viruses in the capripoxvirus genus are cross-protective, meaning vaccination against one will protect against infection by all three [5][6].

The disease caused by the capripox virus is contagious with high morbidity (up to 80%) and mortality (5-50%) viral disease of sheep, goat and cattle leading to substantial economic losses in marginal rural areas of the world. Strains of SPPV do cause disease in goats and some GTPV strains do cause disease in sheep, and some strains appear to cause equally severe disease in both species. The disease inflicts losses in terms of lower quality of wool, leather, and protein loss, reduced output of animal production, increased costs of disease control, reduced milk yield and weight [7][8]. Capripoxviruses have the threats to become potential emerging disease because of global climatic change and due to porous international boundaries which facilitate for migration of animals and animal products from neighbouring countries. Sheep and goat pox is endemic in Africa, parts of Middle East , Europe and Asia. The disease is transmitted by direct contact with infected animals or by contaminated objects [9]. In cattle's Lumpy skin disease (LSD) is a serious significant Transboundry, emerging skin viral disease and it is included in the list of Notifiable Diseases of OIE[10]. Capri poxviruses indicate that these viruses, are the closely related (estimated 96 to 97% nucleotide identity), can be distinguished from one another and may undergo recombination in nature [11]. For these viral specific identification relies exclusively on the use of molecular tools because they are serologically identical. To monitoring the spread of these viruses and controlling outbreaks in susceptible livestock molecular diagnostic tests play an important role [12] [13].

The objective of the research work is the study of Capripox viruses these are the most serious viral diseases of economically important domestic animals it causes high morbidity and mortality rate in sheep and goat industries[14]. p32 one of the major immunogenic genes of Capripoxvirus and it is the major envelop protein corresponds to an envelope protein homologous to P35 protein encoded by Vaccinia virus H3L gene, and locates on the membrane surface of the viral particle [15], [16].Three dimensional structure of Capripox virus major envelop protein p32 Prediction and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

2. MATERIALS AND METHODS

2.1. Protein structure Sequence retrieval:

The *Capripox* viruses major immunogenic P32 protein sequences were retrived from National Center for Biotechnology Information (NCBI) Web address of NCBI is www.ncbi.nlm.nih.gov. The accession numbers are AYI57795.1 is the major envelope protein Sheeppox virus, APY21423.1 envelope protein of Goatpox virus and AAD31773.1 P32 antigen of Lumpy skin disease virus.

2.2. Physico-chemical and secondary structural characterization: The Physico-chemical characterization of Capripox vial major envelop protein was carriedout by using Expasy's ProtParam server (http://us.expasy.org/tools/protparam.html). The computed parameters were include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, instability index, aliphatic index and grand average of hydropathicity (GRAVY). To calculate the secondary structural features of the protein sequence was carried out by using SOPMA tools [17][18][19]. The results were tabulated in Table 1.

2.3. Homology modeling and structural validation: Homology modeling and 3D structural prediction of Capripox vial envelop p32 proteins was carried out by using phyre2,

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(http://www.sbg.bio.ic.ac.uk/phyre2) which uses advanced remote homology detection methods to build 3D models. The evaluation of protein structural models was performed by using PROCHECK, Swiss-PdbViewer software and Finally chosen model was visualized by Rasmol tools[20][21] [22] [23].

2.4. Multiple sequence analysis: The selected protein sequences sequence of Capripox viruses was aligned using online Basic Local Aligment Search Tool (BLAST) andMSA was carried out by using Clustal Omega multiple sequence alignment program at EMBL- EBI that uses seeded guide trees and used to be generate alignments between three or more sequences and to search the mutated region.
(http://www.ebi.ac.uk/Tools/msa/clustalo).

2.5. Smart domain analysis: SMART is a inhouse tool (Simple Modular Architecture Research Tool), as well a growing collection of profiles for shuffled extracellular domains and it allows users to quickly identification and annotation of protein domains and the exploration of protein domain architectures[24][25].

2.6. I-mutant to detect the effect of mutations on the protein Stability: Protein stability was performed by I-MUTANT2.0 online tool (http://folding.biofold.org/i-mutant/i-mutant2.0.html.) it is a Support Vector Machines based Predictor of Protein stability Changes upon Single Point Mutation from the Protein Sequence and Structure. The input and output would be the target site with the changing of amino acid as well the effect of the specified change on the stability of the protein[26][27].

3. RESULTS AND DISCUSSION

3.1. Sequence retrieval: In the present study, the protein sequence of capripox viral p32 was retrieved from NCBI. the selected sequence listed below for further structural prediction and analysis.

>AYI57795.1 major envelope protein [Sheeppox virus]
MADIPLYVIPIVGREISDVVPELKSDNDIFYKKVDTVKDFKNSDVNFFLKDKKDDISLS
YKLLIWEKVEKSGGVENFTEYFSGLCNALCTKEAKSSIAKHFSLWKSYADADIKNSE
NKFIVVIEDDNTLKDSIIIHNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYT
GGYDFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYIL
HNTKYLSKKRANEMKNGIWNRVGKWMAHRFPDFSYYVSHPLVSFFGIFDISIIGALII
LFIIIMIIFNLNSKLLWFLAGMLFTYII
>APY21423.1 envelope protein [Goatpox virus]
MADIPLYVIPIVGREISDVVPELKSGNDIFYKKVDTVKDFKNSDVKFFLKDKKDISLSY
KFLIWEKVEKSGGVENFTEYFSGLCNALCTKEVKSSIAKHFSLWKSYADADIKNSEN
KFIVVIEDDNTLKDLITIYNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTG
GYDFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYILH
NTKYLSKKRANEMKNGIWNRVGKWMAHRFPDFSYYVSHPLVSFFGIFDISIMGALIIL
FIIIMIIFNLNSKLLWFLAGMLFTYIV
>AAD31773.1 P32 antigen [Lumpy skin disease virus]
MADIPLYVIPIVGREISDVVPELKSDNDIFYKKVDTVKDFKNSDVNFFFKDKKDISLSYKFLIW
EKVEKSGGVENFTEYFSGLCNALCTKEAKSSIVKHFSLWKSYADADIKNSENKFIVVIEDDNT
LKDLITIHNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTGGYDFTLSAYVIRLSSA
IKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYILHNTKYLSKKRANEMKNGIWNR
VGKWMAHRFPDFSYYISHPLVSFFGIFDISIIGALIILFIIIMIIFDLNSKLLWFLAGMLFTYII

3.2. Physico-chemical and secondary structure characterization: The computational analysis of physicochemical and secondary structural properties of three envelop p32 proteins of capripox virus was done. the detailed results were presented in Table 1& 2.

Table 1. Physico-chemical characterization Parameters computed using Expasy's

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ProtParam tool.

Property	values of P32 major envelope protein		
	Sheeppox virus	Goatpox virus	Lumpy skin disease virus
Number of amino acides	323	322	322
Molecular weight	37583.58	37530.66	37593.62
Theoretical pl	8.24	8.84	8.24
Total number of negatively charged residues(Asp+Glu)	38	36	38
Total number of positively residues (Arg+Lys)	40	41	40
Extinction coefficient (1.330, assuming all pairs of Cys residues form cystines)	49975	51465	49975
Extinction coefficient* (1.326, assuming all Cys residues are reduced)	49850	51340	49850
Instability index	28.63	27	28.18
Aliphatic index	103.81	101.99	102.61
Grand average of hdropathicity	-0.041	-0.022	-0.03

Table 2. Calculated secondary structure elements by SOPMA.

Property	Value(%) of P32 major envelope protein		
	Sheeppox virus	Goatpox virus	Lumpy skin disease virus
Alpha helix (Hh)	0.96%	1.86%	0.65%
310 helix (Gg)	0.00%	0.00%	0.00%
Pi helix (Ii)	0.00%	0.00%	0.00%
Beta bridge (Bb)	0.00%	0.00%	0.00%
Extended strand (Ee)	19.50%	20.19%	20.19%
Beta turn (Tt)	6.50%	10.87%	5.90%
Bend region (Ss)	0.00%	0.00%	0.00%
Random coil (Cc)	30.03%	17.08%	28.26%
Ambiguous states (?)	0.00%	0.00%	0.00%
Other states	0.00%	0.00%	0.00%

3.3. Protein structure prediction and evaluation: The protein tertiary structure was predected by PHYRE2, this server can perform ab initio modeling to improve models figure. Ramachandran plot of Capripox viral envelop protein generated using Procheck software. The detailed protein structural prediction 3D modelled and validation of proteins results were shown below images.

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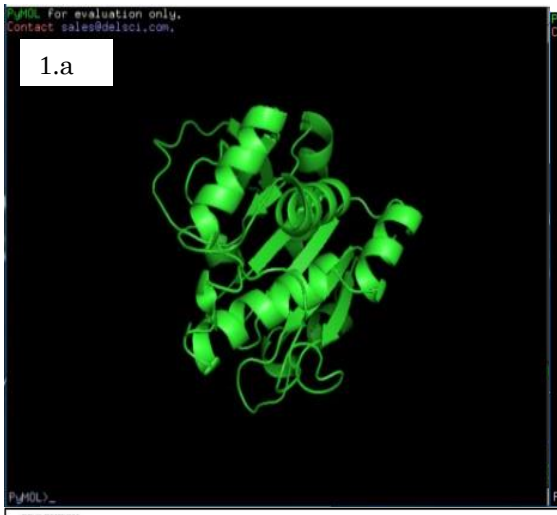

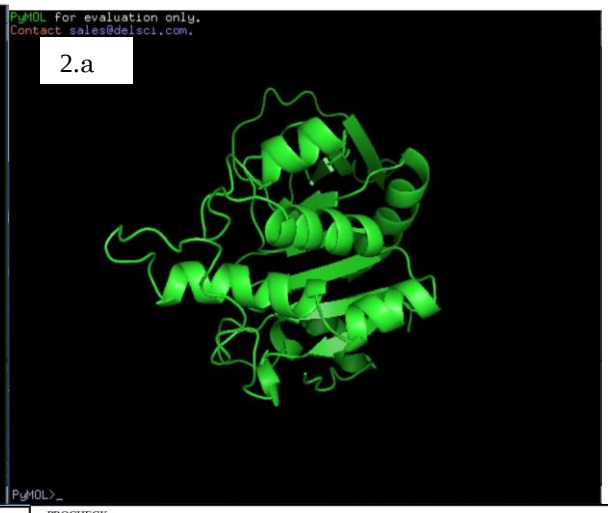

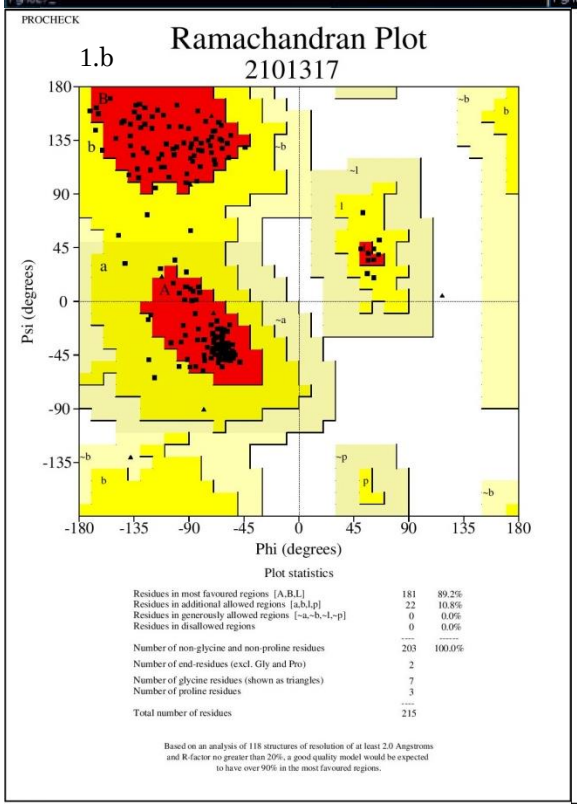

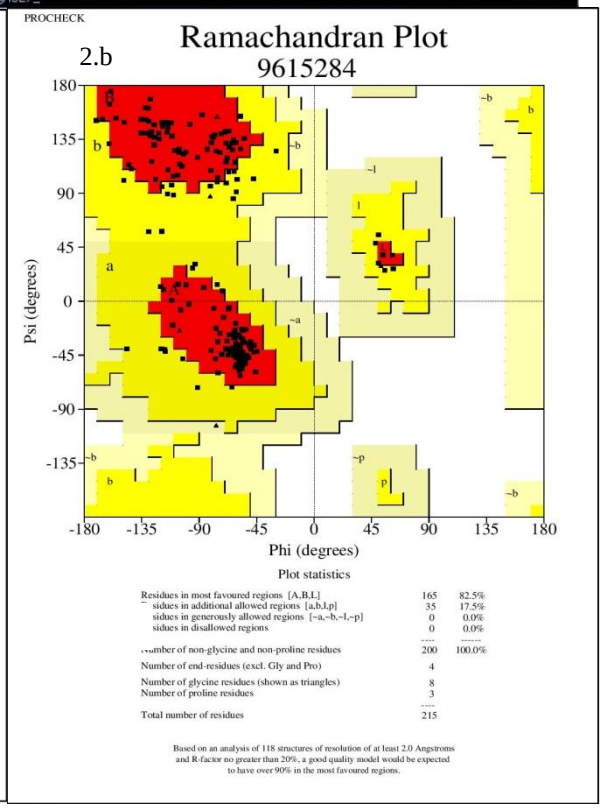

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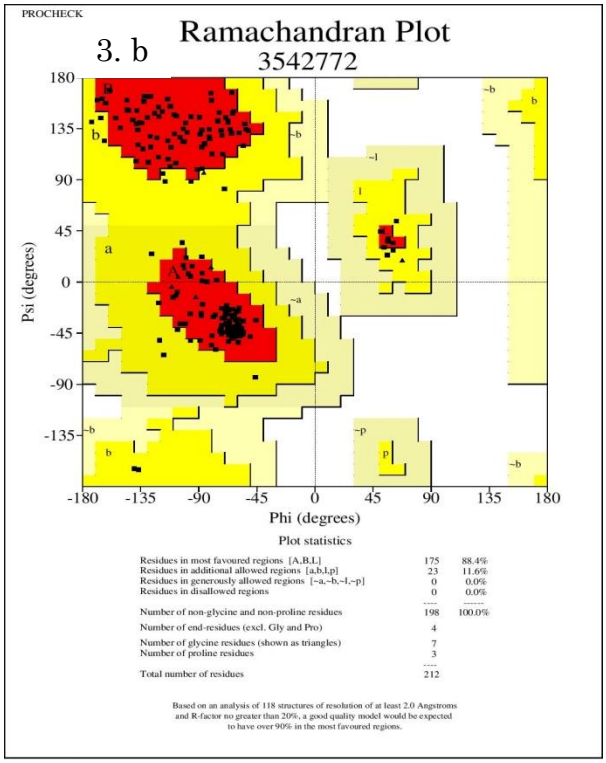

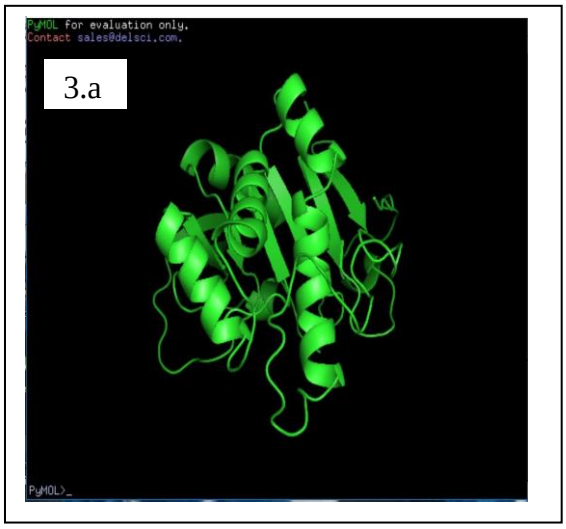

Figure. 1.a and 1.b images are the SPV p32 protein structural prediction 3D modelled and validated by PROCHECK.

Figure. 2.a and 2.b images are the GPV p32 protein structural prediction 3D modelled and validated by PROCHECK.

Figure. 3.a and 3.b images are the LSDV p32 protein structural prediction 3D modelled and validated by PROCHECK.

3.4. BLAST and Multiple sequence analysis:

The BLAST Results of capripoxvirus p32 envelop proteins were shown identies of [Lumpy skin disease virus 315/323(98%) and goatpox virus 313/323(97%) with sheeppox virus. Multiple sequence alignment of protein selected sequences were shown variation at 26 position Aspartic acid aminoacid similar in LSDV and SPV in GPV virus instead of Aspartic (D) acid Glycine (G) is present. 46 aminoacid position Asparagine (N) is similar both LSDV and SPV, SPV envelop protein shown extra Aspartic acid at 54 position where as in LSDV and GPV shown a gap or absent aminoacid at particular position. LSDV and GPV shown similar amino acid at 132, 134 positions where as in SPV at 132 position Serine and 134 position Isoleucine variations was observed. at 290, 325 amino acid position SPV and LSDV were shown Isoleucine but GPV shown Methionine at 290 position and at 322 position Valine variation. SPV and GPV were shown similar amino acid at 305 position where as LSDV Aspartic acid (D) was observed. These results may help to analyse identification species basic envelop based diagnostic marker development.

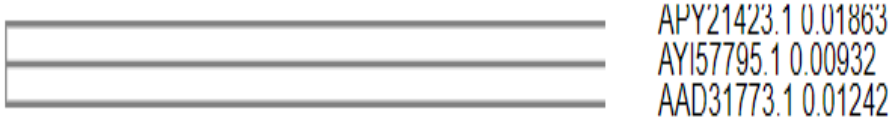

Figure 4: The Phylogenetic variation of Capripox virus envelop p32 Protein

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```
CLUSTAL O(1.2.4) multiple sequence alignment


APY21423.1      MADIPLYVIPIVGREISDVVPELKSGNDIFYKKVDTVKDFKNSDVKFFLKDKK-DISLSY    59
AYI57795.1      MADIPLYVIPIVGREISDVVPELKSDNDIFYKKVDTVKDFKNSDVNFFLKDKKDDISLSY    60
AAD31773.1      MADIPLYVIPIVGREISDVVPELKSDNDIFYKKVDTVKDFKNSDVNFFFKDKK-DISLSY    59
                ************************.******************:**:**** ******

APY21423.1      KFLIWEKVEKSGGVENFTEYFSGLCNALCTKEVKSSIAKHFSLWKSYADADIKNSENKFI    119
AYI57795.1      KLLIWEKVEKSGGVENFTEYFSGLCNALCTKEAKSSIAKHFSLWKSYADADIKNSENKFI    120
AAD31773.1      KFLIWEKVEKSGGVENFTEYFSGLCNALCTKEAKSSIVKHFSLWKSYADADIKNSENKFI    119
                *:****************************.****.**********************

APY21423.1      VVIEDDNTLKDLITIYNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTGGY    179
AYI57795.1      VVIEDDNTLKDSIIIHNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTGGY    180
AAD31773.1      VVIEDDNTLKDLITIHNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTGGY    179
                ********** * *:*****************************************

APY21423.1      DFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYILHNT    239
AYI57795.1      DFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYILHNT    240
AAD31773.1      DFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYKLEKELKLNRQVLNDSSKYILHNT    239
                ************************************************************

APY21423.1      KYLSKKRANEMKNGIWNRVGKWMAHRFPDFSYYVSHPLVSFFGIFDISIMGALIILFIII    299
AYI57795.1      KYLSKKRANEMKNGIWNRVGKWMAHRFPDFSYYVSHPLVSFFGIFDISIIGALIILFIII    300
AAD31773.1      KYLSKKRANEMKNGIWNRVGKWMAHRFPDFSYYISHPLVSFFGIFDISIIGALIILFIII    299
                ********************************:***************:**********

APY21423.1      MIIFNLNSKLLWFLAGMLFTYIV 322
AYI57795.1      MIIFNLNSKLLWFLAGMLFTYII 323
AAD31773.1      MIIFDLNSKLLWFLAGMLFTYII 322
                ****:*****************:
```

Figure 5: Multiple sequence analysis of selected p32 protein sequences

3.5. Smart domain analysis:

The capripox viral p32 envelop protein domain identification was performed using the SMART online tool the result were shown below the tables. The outlier homologues and homologues of known structure SPV shown three scop domain was charactise of Structural Classification of Proteins d1a9xb2- Superfamily: Class I glutamine amidotransferase-like, d1i1ga2-Superfamily: Dimeric alpha+beta barrel and d1ffta1-Protein: Cytochrome O ubiquinol oxidase, subunit I from Escherichia coli. GPV shown only one Superfamily: Phosphoenolpyruvate/pyruvate domain and LSDV shown two structural classification d1k42a_- Superfamily: Galactose-binding domain-like d1flga_- Superfamily: Quinoprotein alcohol dehydrogenase-like classifications.

Table 3. Confidently predicted domains, repeats, motifs and features:

Name	Start	End	E-value
SPV_p32_Pfam:Pox_P35	1	323	6.3E-136
GPV_p32_Pfam:Pox_P35	1	322	3.4E-136
LSDV_p32_Pfam:Pox_P35	1	322	3.3E-136

Table 4. Outlier homologues and homologues of known structure:

Name	SCOP domain	Sequence	Start	End	e-value
SPV_p32_Pfam:Pox_P35	d1a9xb2	LCNALCTKEAKSSIAKHFSLWKSYADADIKNSENKFIVVIED	83	125	4.2
	d1i1ga2	SYTGGYDFTLSAYVIRLSSAIKIINEIIKNKGISTSLSFEMYK	174	217	6.6

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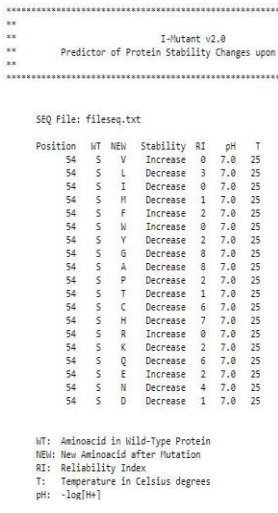
	d1ffta1	AHRFPDFSYYVSHPLVSFFGIFDISIIGALIILFIIIMIIFNL	263	306	5.2
GPV_p32_Pfam:Pox_P35	d1e0ta2	IVGREISDVVPELKSGNDIFYKKVDTVKDFKNSDVKFFLKDKKDISLSYKFLIWEKVEKSGGVENFTE	10	78	2
LSDV_p32_Pfam:Pox_P35	d1k42a_	SYKFLIWEKVEKSGGVENFT	57	77	2.8
	d1flga_	ENKFIVVIEDDNTLKDLITIHNIIIEMQEKNIDIFQLRETFHNSNSRILFNQENNNFMYSYTGGYDFTLSAYVIRLSSAIKIINEIIKNKGISTSLSF	114	212	3.5

3.6. I-mutant to detect the effect of mutations on the Stability: Based on protein silmilarity and Capripox viral envelop protein SPV shown one extra amino acid at 54 Aspartic acid where as in LSDV and GPV is absent the present study I-mutant was carried out at 54 position of protein sequence of the viral protein which is important immunogenic protein. I-Mutant2.0 predictions are performed starting from the protein structure or, from the protein sequence used to analyse the p32 enveolp protein of capripox virus. the Prediction of the direction of the protein stability changes upon single point mutation only from the protein sequence detect the effect of all the other 19 combinations of amino acids at the 54 position on the stability of the protein.The results of mutation analysis using I-Mutant2.0 predictions was shown the possibility of structure stability decreasing this can conclude that the 54 position is more important according to the stability and functionality and important site for the study. The detailed protein structure stability changing of 19 aminoacid at 54 position results were shown image.



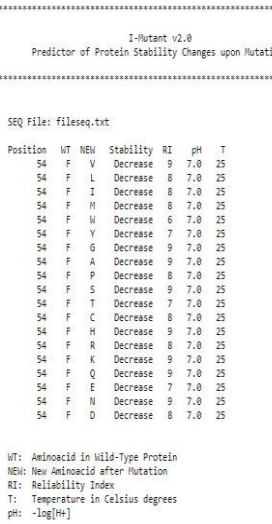
I-Mutant v2.0
Predictor of Protein Stability Changes upon Mutations

SEQ File: fileseq.txt

Position	WT	NEW	Stability	RI	pH	T
54	S	V	Increase	0	7.0	25
54	S	L	Decrease	3	7.0	25
54	S	I	Decrease	0	7.0	25
54	S	M	Decrease	1	7.0	25
54	S	F	Increase	2	7.0	25
54	S	W	Increase	0	7.0	25
54	S	Y	Decrease	2	7.0	25
54	S	G	Decrease	8	7.0	25
54	S	A	Decrease	8	7.0	25
54	S	P	Decrease	2	7.0	25
54	S	T	Decrease	1	7.0	25
54	S	C	Decrease	6	7.0	25
54	S	H	Decrease	7	7.0	25
54	S	R	Increase	0	7.0	25
54	S	K	Decrease	2	7.0	25
54	S	Q	Decrease	6	7.0	25
54	S	E	Increase	2	7.0	25
54	S	N	Decrease	4	7.0	25
54	S	D	Decrease	1	7.0	25

WT: Aminoacid in Wild-Type Protein
NEW: New Aminoacid after Mutation
RI: Reliability Index
T: Temperature in Celsius degrees
pH: -log[H+]

Figure: 6.a. SPV p32



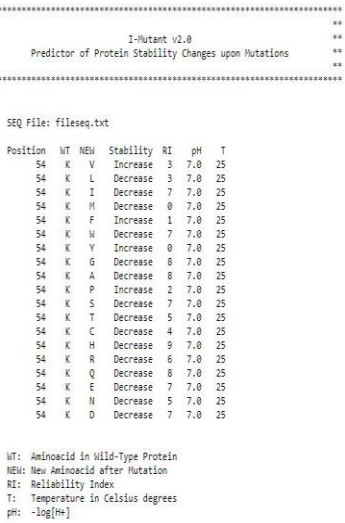
I-Mutant v2.0
Predictor of Protein Stability Changes upon Mutations

SEQ File: fileseq.txt

Position	WT	NEW	Stability	RI	pH	T
54	F	V	Decrease	9	7.0	25
54	F	L	Decrease	8	7.0	25
54	F	I	Decrease	8	7.0	25
54	F	M	Decrease	8	7.0	25
54	F	W	Decrease	6	7.0	25
54	F	Y	Decrease	7	7.0	25
54	F	G	Decrease	9	7.0	25
54	F	A	Decrease	9	7.0	25
54	F	P	Decrease	8	7.0	25
54	F	S	Decrease	9	7.0	25
54	F	T	Decrease	7	7.0	25
54	F	C	Decrease	8	7.0	25
54	F	H	Decrease	9	7.0	25
54	F	R	Decrease	8	7.0	25
54	F	K	Decrease	9	7.0	25
54	F	Q	Decrease	9	7.0	25
54	F	E	Decrease	7	7.0	25
54	F	N	Decrease	9	7.0	25
54	F	D	Decrease	8	7.0	25

WT: Aminoacid in Wild-Type Protein
NEW: New Aminoacid after Mutation
RI: Reliability Index
T: Temperature in Celsius degrees
pH: -log[H+]

Figure: 6.b. GPV p32



I-Mutant v2.0
Predictor of Protein Stability Changes upon Mutations

SEQ File: fileseq.txt

Position	WT	NEW	Stability	RI	pH	T
54	K	V	Increase	3	7.0	25
54	K	L	Decrease	3	7.0	25
54	K	I	Decrease	7	7.0	25
54	K	M	Decrease	0	7.0	25
54	K	F	Increase	1	7.0	25
54	K	W	Decrease	7	7.0	25
54	K	Y	Increase	0	7.0	25
54	K	G	Decrease	8	7.0	25
54	K	A	Decrease	8	7.0	25
54	K	P	Increase	2	7.0	25
54	K	S	Decrease	7	7.0	25
54	K	T	Decrease	5	7.0	25
54	K	C	Decrease	4	7.0	25
54	K	H	Decrease	9	7.0	25
54	K	R	Decrease	6	7.0	25
54	K	Q	Decrease	8	7.0	25
54	K	E	Decrease	7	7.0	25
54	K	N	Decrease	5	7.0	25
54	K	D	Decrease	7	7.0	25

WT: Aminoacid in Wild-Type Protein
NEW: New Aminoacid after Mutation
RI: Reliability Index
T: Temperature in Celsius degrees
pH: -log[H+]

Figure: 6.c. LSDV p32

Figure. 6 a, 6b and 6c of capripoxviral p32 envelope protein showing I- Mutant to detect the effect of substitutions on the stability of the protein.

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4. CONCLUSION

Capripox viruses are the most serious viral diseases of economically important domestic animals it causes high morbidity and mortality rate in sheep and goat industries. In this study we predicted three dimensional structure of capripox virus major envelop protein p32 and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

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EPIDEMIOLOGY OF CHILDHOOD LEPROSY IN INDIA: A PILOT SURVEY

Padmaja Deore[1],Ligi Milesh[1]**,Sonika Rai[1], Sabnam Sharma[1], Sanjana Dey[1], Gajanan Gaikwad[1]
[1]Department of Biotechnology, School of Chemical & Biological Sciences, REVA University, Bangalore

ABSTRACT

*Mycobacterium leprae*causes dreadful disease known as leprosy which affects skin, eyes, nasal and peripheral nervous system. The most common symptoms are Paucibacillary (borderline tuberculoid –leprosy) with skin lesion and deformity. These pathogens are mainly transmitted by contacts. Childhood leprosy is predominant in the age group of 10-16 years of age with the reports of clinico-epidemiological pattern of childhood leprosy over the past two decades during the years 1990-2009. It was found that there is high frequency of type 1 lepra reaction. Thus based on predesigned format-demographic and disease characteristics such as age, sex, contact history, period of infection, bacteriological and histopathological conditions, reactions and disabilities various type of studies have been done and noted. Even after the present treatment, frequent occurrence of relapse was observed. Statistical analysis showed that the percentage of childhood cases in India remained same (range 9.42-10.14%) from the year 2005 to 2012. Thus preventive diagnostic and epidemiological approach is required for eliminating childhood leprosy in India.

KEYWORDS: *Mycobacterium leprae*, childhood leprosy, Paucibacillary, Multibacillary, lepra reaction.

****Corresponding author:** Dr. LigiMilesh, Assistant Professor
Department of Biotechnology,REVA University, Bangalore.
Email Address: ligimilesh81@gmail.com

1. INTRODUCTION

Leprosy is a long-lasting, curable contagious disease which is triggered by *Mycobacterium leprae* or *M.lepromatosis*. It causes skin lesions, eyes, nasal and outlying nervous system damage. *Mycobacterium leprae* is a pleomorphic, intracellular, acid fast pathogenic aerobic bacillus bacterium. It can be diagnosed by using carbol fuchsin stain. Norwegian physicians Gerhard armauer Hansen was first to discover *Mycobacterium leprae* bacterium (1873) [1]. In 1983 the prevalence rate of leprosy was found to be 57.8/10,000. However, the effort of National Leprosy Eradication Programme supported by WHO, ILEP and few other NGOs, India succeeded bringing the leprosy rate down to 1/10,000 in December 2005 with the administration of MDT (Multi Drug Therapy). Further,the leprosy rate was brought down to 0.66/10,000 in 2016. Although, leprosy is more common among the adults it also affects the children especially between the age group of 10-15 [2]. The children of one year of age have also been observed with leprosy condition. As per WHO epidemiological record (2012), India had 58% of leprosy cases detected worldwide. Even though the average national child leprosy rate was approximately 9% in 2016 according to Leprosy Case Detection Campaign, 11 states of India showed greater than 10% of cases with very high rate ranging from 14-23% [3].One of the reasons for leprosy being still in the picture, especially in a country like India is also due to the fear of discrimination and stigma in the society and lack of awareness about the disease. It affects almost 700,000 individuals per year [4]. In addition to MDT, other treatments such as chemoprophylaxis and immunoprophylaxis are being considered to be effective against leprosy [5].

2. TRANSMISSION

Leprosy, which is trigerred by the organism *Mycobacterium leprae* is believed to enter the

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humans through the intact lining of the nose and wherever there is any break or damage in the skin but not directly through the lungs, mouth or the digestive tract. This disease can be transmitted from infected ones to the healthy ones if they are having prolonged close contact. The reason why this bacterium has higher incidence in skin, peripheral nerves, testicles and upper airways is because of the temperature of 27^{o}Cand 30^{o}C required for their growth and proliferation. Even though the genes involved in the cause of leprosy is not known fully, it has been found that both the genes of human leukocytes i.e., Ag (HLA) and the non-HLA are associated with the predisposition of this disease [6].

3. LEPRA REACTION

Patients with leprosy develop an immunological disorder and this is called a lepra reaction. Patients with this kind of reactions are prone to disabilities and deformities compared to those with undeveloped reaction [7]. PAL can develop the lepra reaction but some are more likely to suffer than patients with few skin lesions and no swollen nerve and they have the less chance of developing reactions [8]. Leprosy reaction occurrence is unpredictable, it can occur during the course of treatment or after fulfillment of the treatment [9]. The reaction is break down into type-1 and type-2. The lepra 1 reaction is unpredictable with no symptoms and mainly affects skin and nerves. It develops in patients having borderline disease which is the transition between tuberculoid and lepromatous leprosy. A type 2 lepra reaction causes an acute immune complex vasculitis which affect skin and other organs and this reaction is poorly understood [10].

4. DIAGNOSIS

The ongoing transmission and High endemicity in the country were seen; many hidden cases in community showed no change in the rate of new cases detection. Disability rates in new cases were rising due to delay in diagnosis. NLEP advocated 3 prolonged approaches to avoid these and to diagnose the leprosy rate. Leprosy Case Detection Campaign in high enzootic districts, enthralled leprosy awareness through health co-ordinators and ASHA and made area specific plans for case detection. Some of the methods used for the diagnosis of leprosy are Skin Smear Baciloscopy and biopsy. For the children aged 10 years, thermal sensitivity test is tough to be administered. The diagnosis could be therefore complemented by Skin Smear Baciloscopy and biopsy. Multibacillary leprosy can be identified by using skin smears or skin tissues that show acid-fast bacilli with the Ziel-Neelsen stain [11].In addition to MDT, to reduce the chain of transmission and reach the zero status Chemoprophylaxis and Immuno prophylaxis are being considered [12]. Leprosy, PB and MB are treated by using different drugs [13].

5. MULTIDRUG THERAPY REGIMEN

Different drugs are given at different dosage level according to the weight of the child. Some of the drugs used are Dapsone, Rifampicin, Clofazimine, these are given at 1.5,1,10 mg/kg/day respectively. Capsules of rifampicin (15mg), Clofazimine (50mg) and Dapsone (50mg) are given for blisters of both Paucibacillary and multibacillary. Another alternative treatment called oral Corticotherapy showed good impact in the treatment of leprosy. Especially, the Predinose at a prescription of 1mg/kg/day could be used until the analytical conditions come to normal and the gradual removal of the medication.

6. CHEMOPROPHYLAXIS OF CONTACTS

Post exposure chemoprophylaxis (PEP) is a medical treatment which is started instantly after the exposure to pathogen. It helps to stop the infection and simultaneously the occurrence of other clinical diseases. Undiagnosed leprosy patients are rare in the general population compared to the household contacts of leprosy patients. Thus the main aim of global leprosy control strategy is to assess the value of Chemoprophylaxis. Some studies showed the risk reduction in the preventing of development of leprosy using Chemoprophylaxis with Single

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Dose Rifampicin (SDR). Overall 57% risk reduction was seen within first two years of administration. SDR's protective effect were prominent in first 2 years with no additional effect even after 4 to 6 years.

Leprosy Post exposure chemoprophylaxis (LPEP) was launched globally various leprosy stakeholders and agencies in 2014. To generate the evidence for most efficient way to operationalize contact, the treatment will be continued till 2018. The contact tracing is done via post exposure prophylaxis and its potential to interrupt leprosy transmission.

7. SURGERY

In some cases of neuritis the motor function of the involved nerve shows no improvement despite the treatment of leprosy with steroids for 2-4 weeks. Nerve decompression is a surgery where nerve pressure is relieved. It restores the nerve function and relieves nerve pain.

Other treatments include giving reassurance to the patients that medicine would work and they would be fine in few days. Analgesics and anti-inflammatory agents such as Aspirin (600mg up to six times) and Paracetamol (1 gm up to 4 times) can be used to relieve pain.

8. EPIDEMIOLOGY AND STATISTICAL ANALYSIS OF CHILDHOOD LEPROSY IN INDIA

Random studies revealed that 8.94% of children were affected with childhood leprosy in India (NLEP) [14]. Moreover, 5.1-11.43% of occurrence were reported at a tertiary care Hospital in Southern Rajasthan [15-16]. It was also found that the disease condition was relapsing after treatment which ranges from 1.16-7.1%. Statistical analysis showed that the percentage of childhood cases in India persisted same (ranging from 9.42-10.14%) from the year 2005 to 2012 [WHO report (2014)].

Table 1: Occurrence of childhood leprosy at tertiary care hospital in Rajasthan [17].

Sl. no.	Cases	Percentage of occurrence
1	Slit-skin smear positive	5.42-25%
2	*Lepra reactions*	0-29.7%
3	Deformity	0-24%

9. STATISTICAL ANALYSIS

Overall percentage of childhood leprosy in India remained same from the year 2005-2012 i.e, (9.42-10.14%) (WHO report, 2014). Clinico-epidemiological data from various Indian states on childhood leprosy were studied by different authors. State-wise analysis showed that Leprosy Research and Training Centre, Tamil Nadu reported high rate with single lesion BT (80%) (Selvasekar et al., 1999); followed by Tertiary care Hospital, New Delhi with BT (73%) (Sardana K, 2006); followed by Urban clinic, New Delhi with BT (70.8%) (Grover et al., 2005); followed by Tertiary care Hospital, New Delhi with BT (70.3%) (Singal et al., 2011); followed by Community survey, Maharashtra with Single lesion BT (69%) (Shetty et al. (2013); followed by Tertiary care Hospital, Andhra Pradesh with BT (68.75%) (Rao AG, 2009); followed by Urban clinic, Andhra Pradesh with BT (66.3%) (Jain et al., 2002); followed by Leprosy Mission Hospital, West Bengal with TT (43.7%) (Horo et al., 2010); followed by Tertiary care Hospital, Gujarat with BT (35.82%) (Vara N, 2006).

Table 2. State-wise analysis of childhood leprosy in India [17].

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Sr.No.	Set-up/study of location	Number of childhood leprosy cases detected (%)	Age group (years)	Commonest clinical type (R-J classification)
1	Tertiary care hospital, Tamil Nadu	n=66(7.2)	0-14	Not specified
2	Leprosy Research & Training centre, Tamil Nadu	n=794(31.3)	0-14	Single lesion BT (80%)
3	Urban clinic, Andhra Pradesh	n=306(9.81)	0-14	BT (66.3%)
4	Urban clinic, New Delhi	n=137(7.06)	0-14	BT (70.8%)
5	Leprosy Mission Hospital, West Bengal	n=258(18)	10-20	Not specified
6	Tertiary care Hospital, New Delhi	n=86(7.71)	0-15	BT (73%)
7	Tertiary care Hospital, Gujarat	n=67(8.4)	0-14	BT (35.82%)

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8	Tertiary care Hospital, Andhra Pradesh	n=32(11.43)	0-18	BT (68.75%)
9	Leprosy Mission Hospital, West Bengal	n=151(not quoted)	0-15	TT (43.7%)
10	Tertiary care Hospital, Uttar Pradesh	n=219(5.1)	0-15	Not specified
11	Tertiary care Hospital, New Delhi	n=172(9.6)	0-14	BT (70.3%)
12	Community survey, Maharashtra	n=32(R) (25.5) n=36(U) (33)	0-14	Not specified (R),Not specified,(U),Single lesion BT 69%

R=Rural, U=Urban

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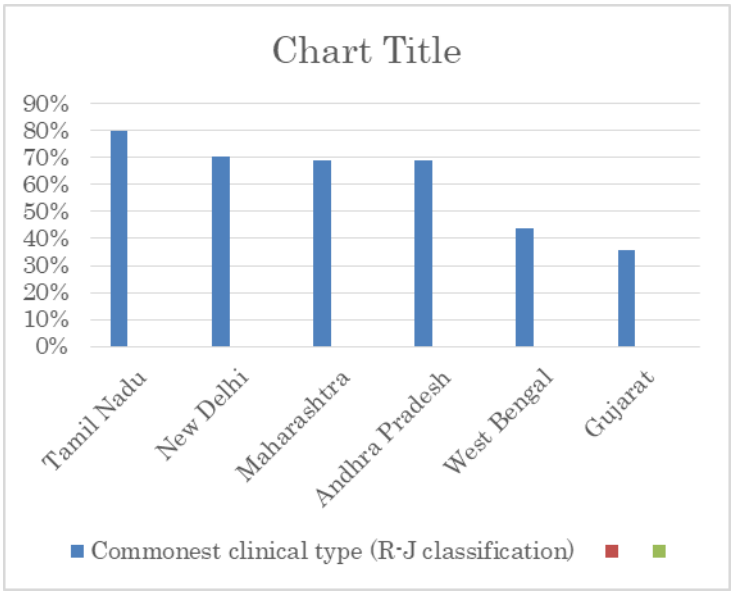

Figure 1: State-wise analysis of childhood leprosy.

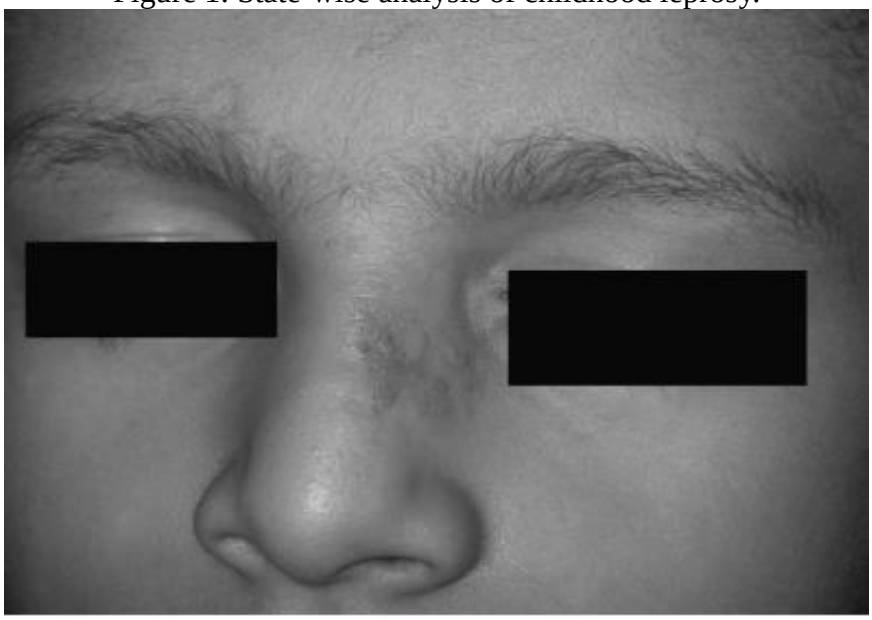

Figure 2: Single erythematous plaque of BT leprosy appeared on the face of a child (8 year old) [17].

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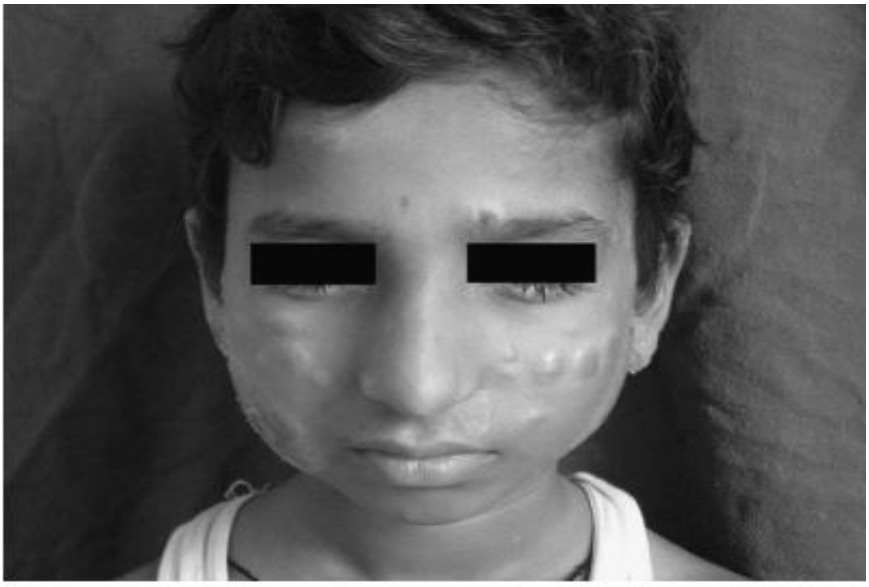

Figure3: A 13 year old boy with BL leprosy having type 1 reaction. Edematous and scaly plaques are visible on the skin [17].

10. CONCLUSION

Multibacillary cases occur frequently among Indian children in contrast to the typical concept that it is rare in children. So children may show smear positive test & can be a source for many other cases. Familial and extra-familial contact may be the cause for greater occurrence of childhood leprosy cases as per some of the authors. These contacts may contribute in the present situation of childhood leprosy in India. These studies has statistical records as well as epidemiological significance. These studies leads to more intense community survey for detecting existence of childhood leprosy. Socio-economic factors are also responsible for influencing leprosy-related deformity in children. Illiteracy, ignorance, financial conditions of some of the families are the contributory factors for leprosy-related deformities. BCG vaccination awareness should be conveyed through certain campaigns, mainly in high endemic states as well as in families havingleprosy backgrounds. The main aim of discussing the above mentioned statistical research in India is to know the current status of childhood leprosy when the nation is at the verge of eradicating leprosy. Through television & newspapers awareness can be made about the disease to overcome the tragedy.

Although treatment has shown good sign of recovery, reports have showed relapse & reoccurrence of disease which threat to the public. It is the area of concern among the huge population. Thus, the present article of preventive, diagnostic, epidemiological & statistical analysis would help eliminating childhood leprosy from India.

11. DISCUSSION

From the data on childhood leprosy as mentioned above statistical analysis has been done (Table 2). Some studies were conducted in tertiary care hospitals. Patients, parents and other referrals voluntarily reported. General community surveys, school surveys were the modes of detecting cases in urban areas. 5.1-11.43% of occurrence were reported at a tertiary care Hospital as mentioned in the (Table 2).

The clinical type-borderline tuberculoid (BT) was recorded as the commonest single lesion disease. The occurrence of peripheral nerve trunk were observed commonly. Various studies reported that Paucibacillary cases ranged from (43.28%-98%) whereas (2%-56.6%) cases were reported for multibacillary. According to some of the studies, familial contacts ranged from (0.66%-47%). Parents, grandparents & siblings are the modes of contact. These contacts reported smear positivity for Multibacillary in some of the cases. According to the two

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studies, occurrence of non-familial contacts were 1.96% (Jain et al.) & 2.9% (Singal et al.). High percentage of childhood leprosy ranging from (62%-69.6%) were observed as reported by two studies conducted at New Delhi. These children belonged to immigrant families who came from neighboring states. These states were endemic for leprosy. Relapse varying from 1.16%-7.1% was recorded as per 3 studies. According to the WHO report, most of the studies showed deformity based on disability grading. Some of the authors found that BCG offers protection against leprosy. BCG vaccination proved to partially halt the transmission of the disease.

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"THE VARIATION IN THE GLUCOSE LEVELS AFTER A NIGHT SHIFT TO A CASUAL DAY: A CROSS OVER TRAIL IN HEALTHY VOLUNTEERS"

Daisy K L[1], V Veeraraghavan[1]

[1]School of Biochemistry, REVA University, Rukmini Knowledge Park, Kattigenahalli, Yelahanka Bangalore – 560064, Karnataka, India.

ABSTRACT: A well regulated glucose metabolism and glucose homeostasis are seen in every healthy individual. The body responds to the high and low levels of glucose by regulating various anabolic and catabolic reactions. It is also regulated by the circadian rhythm. This study aims at looking into the probable changes of this regulation in healthy volunteers after their night shift. Fasting blood glucose levels are obtained from healthy volunteers after their night shift and on a regular day and the results are compared. During night shifts the body's biological clock is disrupted and there is change in the glucose levels in comparison with the ordinary days in which one had a good night's rest. An attempt is made to see the stress & fatigue levels (cortisol) on a night shift and on a regular day and its impact in the variations of glucose levels. Whenever one is on stress hormone levels go high, this will in turn activate various sources energy into the cells. Hence there is an increase of glucose levels in the blood. Here a similar working atmosphere is given for the both shifts. Exclusion criteria is made by taking into account the pre disposing factors that can influence the results and the target age group is between 20-40. Considering all above mentioned factors the report gives apparent understanding that the disruption in the biological clock causes metabolic changes in every individual.

KEY WORDS: Corticosteroid hormone (Cortisol), Glucose homeostasis, Circadian rhythm

***Corresponding author:** Daisy K L,School of Biochemistry, REVA University, Email: srdaisyjmj@gmail.com

1.INTRODUCTION

Lot of physiological and chemical changes occurs in the human body based on the circadian rhythm. It consists of a constant biological oscillation of 24 hours that present in all living systems. It is a time keeping system influenced by molecular make up and not just alone the response to changes in the day and night circles and is a "biological clock"[1,2]. In the suprachiasmatic nuclei (SCN) of the hypothalamus the circadian clock is situated and it acts in response to the signals of the light/dark cycle in the external environment. There is an information that is to the hypothalamus from the retina [3] the retino-hypothalamic pathway, in response to this signal the SCN synchronize the biological clock [4,5]. Glucose metabolism is highly influenced by this rhythm of the body, various metabolic pathways gets activated according to the time of the day [6]. When one is awake or night shift, especially those working in a rotating shift schedule, are constantly changing their sleep/wake and feed/fast cycle. In normal days this biological clock makes sure that all the metabolic cycles take in a regular rhythm [7-9]. Lack of adequate sleep, irregular eating patterns, exposure to light all these can affect the circadian function [10,11] and negatively influence glucose metabolism [12]. The people on different shift duties are prone to be having change in this normal pattern, there is change in their rhythm to which the body is trying to adopt and change.

Cortisol is a primary catabolic hormone and is one of the steroid hormones known as glucocorticoids. It is synthesized and secreted from the adrenal cortex and a small amount derived from cortisone. Cortisol decrease protein synthesis and increase protein degradation [13]. On a regular day the cortisol secretion increases in the second half of the night and

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reaches maximum at the early hours of the day. After this the levels tend to remain low and the lowest at first half of the night [14]. But it is also noted that cortisol is at the peak after 30 minutes of awakening, it can be increased up to 60 minutes after waking [15]. This is comparatively constant for any individual [15, 16].

In this study an attempt is made to examine the glucose metabolic variations in individuals on night shift and otherwise. It also aimed to confirm the temporal difference of fasting glucose and body's metabolic response to this change in the circadian clock in healthy individuals. Cortisol levels are analyzed to identify the probable stress and fatigue during the night shift and its influence in the glucose metabolism.

2.MATERIALS AND METHODS

2.1 Participants:-

Eligible volunteers were healthy males and females aged between 20 and 40 years. Those diagnosed with diabetes mellitus, cardiovascular disease or sleep disorders; taking oral hypoglycemic agents, antihypertensive or lipid-lowering medication were excluded. Participation was voluntary; written informed consent was obtained from all participants.

2.2 Study design

The Study includes two separate cross trails. The first trial is employed to do the glucose levels in the morning on a regular day at 8 am. The individuals are requested to come in fasting. In the second trial is done after a night shift. The study was conducted at St. Philomena's Hospital Bangalore. And all the volunteers were staff nurses and were staying at the hostel, hence the probable glycemic index of the food consumed were similar. The volunteers were asked not to eat or chew anything during their night duty, after 10 pm.

2.3 Assay proper

Plasma glucose was analyzed using *Cobas Interga* Chemistry Auto analyzer as per manufacturer instructions. Cortisol levels were obtained in chemiluminscence analyzer *acess-2 of Beckman Coulter*.

2.4 Statistical Methods:

Descriptive statistical analysis has been carried out in the present study. Results on continuous measurements are presented on mean and results on categorical measurements are presented in number. Significance is assessed at 5% level of significance. Student t test has been used to find the significance of study parameters on continuous scale within each group.

Student t-test for paired comparisons

Objective: To investigate the significance of the difference between single population means. No assumption is made about the population variances.

3.RESULTS

In the results obtained it is evident that the glucose levels is higher during the night shift in comparison with the normal day. The cortisol levels are lower on the night shift and higher on the normal day. None of the cortisol results crossed the REFERENCES range. It is evident that the change in the biological clock will bring about a certain changes in the metabolic activities in the body.

Table 1: The results obtained are given in table below.

S.No	Glucose levels		Cortisol levels	
	After the night shift	Normal day	After the night shift	Normal day
1	86	82	9.22	12.8
2	106	100	10.55	17.32

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3	99	90	17.08	18
4	112	97	9.18	14.67
5	93	91	7.49	21.1
6	103	95	10.78	15.16
7	100	96	9.59	20.72
8	94	88	8.55	8.71
9	97	96	7.17	8.1
10	100	96	12.21	15.31
11	107	99	8.48	10.2
12	84	86	10.66	17.48
13	92	88	6.82	8.92
14	88	86	8.74	12.6
15	102	95	8.29	9.01
16	110	105	15.45	15.96
17	85	82	8.9	10.12
18	105	100	11.56	13.8
19	98	91	8.73	9.18
20	109	96	10.59	20.19

Graph 1: The graph below is a comparison between the glucose levels on both days. FBS -1 is done after the night shift and FBS-2 on regular day.

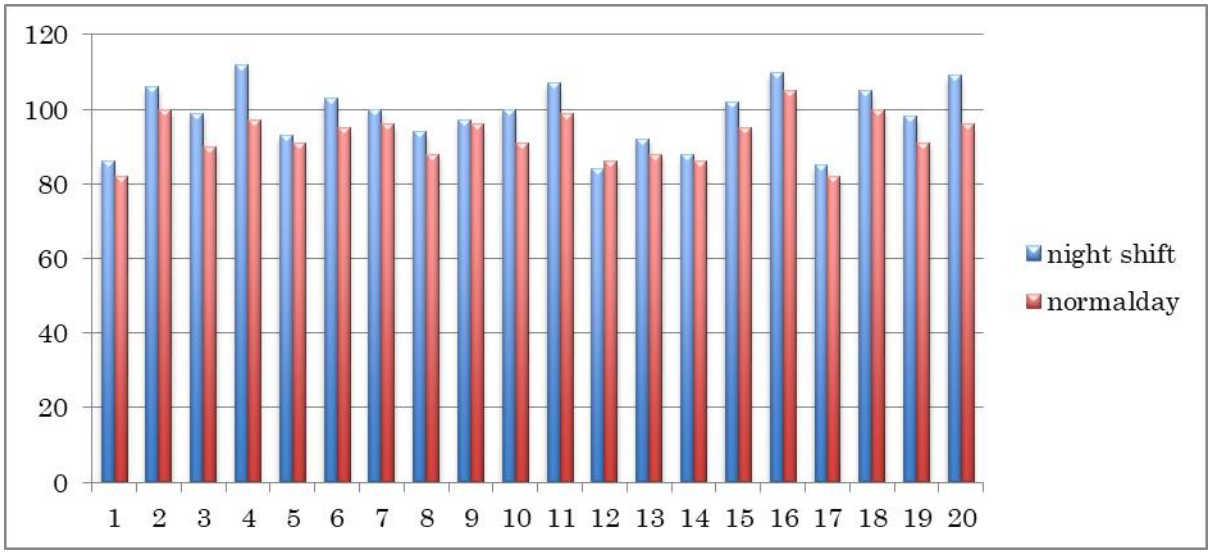

Table 2: Levels of glucose on 2 different days

Glucose	Range	Mean
Normal Day	82 – 105	92.7
After night shift	86 – 112	98.5

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Graph 2: The cortisol levels are plotted below in the graph. Cortisol -1 and 2 are the results obtained after night shift and regular day respectively.

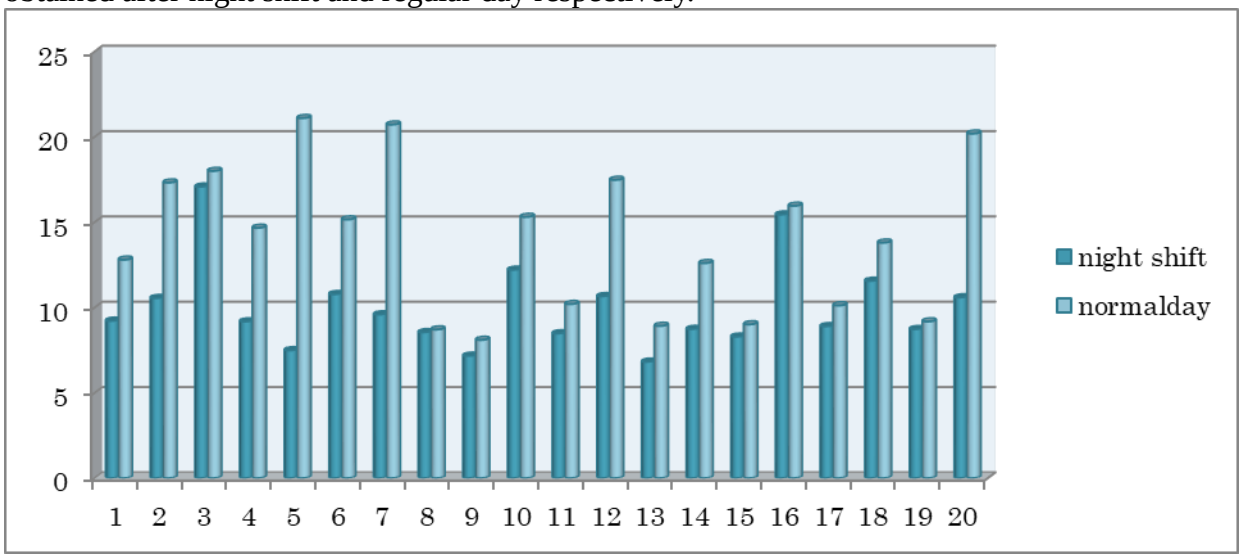

Table 3: Levels of Cortisol on 2 different days

Cortisol	Range	Mean
Normal Day	8.1 – 20.72	13.9675
After night shift	6.82 -17.08	10.002

4. DISCUSSION

The study consists of samples obtained on two different days (normal day Vs after the night shift) are undertaken for the study. The objective of the study is identifying the changes in the glucose levels due to the interruption to the circadian rhythm in healthy individuals. The glucose values are tending to remain higher in the night shift than the normal day. 99% of the subjects had the same food for the dinner and the glycemic index almost is the same. There are lot of studies being carried out since 1960's about the diurnal variations in glucose tolerance and the evidence of circadian regulation of glucose metabolism[17- 21]. The fasting glucose is high in the morning in comparison with any part of the day [22, 23]. Another fact is the intermittent consumption of snacks and drinks (coffee or tea) can influence this increase. The cortisol levls are low after night shift in comparison with the regular day. This phenomen can be due to fatigue [24] work demand in the night. The cortisol levels are high on an ordinary day after the night's rest. It is been noticed that the cortisol awakening response is high when one gets up to a working day contrast to a day off or free weekend [25, 26].

5. CONCLUSION

It is evident that the change in the biological clock will bring about a certain changes in the metabolic activities in the body. There are various things to be looked into, the increase in glucose levels after night shift can be due to various factors. The usual dawn effect (rise in glucose levelsin the early hours of the day 2 am – 8am) is unlikely because the nocturnal hormones are not increased due to the disruption in the bilological clock. The cortisol levels

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are low at night shift is a clear indication for the above mentioned phenomen. Tiredness in the night Vs huzzle and buzzle of the ordinary day can be the reason for the changes in cortisol levels. Furthermore it is clear that biochemical investigations that are performed after night shift or improper rest vary from the actual results.

CONFLICT OF INTEREST

None

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IN-SILICO ANALYSIS AND HOMOLOGY MODELING OF INTRACELLULAR MATURE VIRION A28 PROTEIN OF CAPRIPOX VIRUSES ALONG WITH VACCINIA VIRUS.

[1]KiranKumar Burranboina,*[1,3]Kumar KM, [2]Manjunatha Reddy G B, [2]Yogisharadhya R, [3]sunil abraham

[1]Department of Biotechnology, REVA University, Bengaluru, India.
[2]National Institute of Veterinary Epidemiology and Disease Informatics, Bengaluru, India
[3]Dayananda Sagar college of engineering, Bengaluru, India.

ABSTRACT: Capripox diseases are notifiable to Office International des Epizooties and enzootic in different country parts. which causes significant economic losses by affecting domestic animals And have the threats to become potential emerging disease because of global climatic change and due to permeable international boundaries which facilitate for migration of animals and their products from neighbouring countries.A28 is the membrane component of the intracellular mature virion,during the virus replication cycle the poxvirus A28 protein is expressed at late times and A28-dependent mechanism of cell penetration commonly use and suggesting that by all poxviruses. The results of this study Structure Prediction, identification of SMART domain sequences of fusion protein A28 capripox virus along with shown similar properties except vaccinia virus.The SMART analysis vaccinia virus shown the SCOP:d1epxa represents Superfamily: Aldolase. These models gives is stable structural information of this model can be effectively understand used in evolutionary relationship between the closely related Capripox viruses with vaccinia virus in future to develop recombinant vaccine, vector and drug designing.

Keywords: Intracellular Mature Virion A28,Capripox virus,Vaccinia virus,Homology modelling.

***Corresponding Author:** Dr. Kumar KM, Department of Biotechnology, REVA University, Bengaluru, India. E. mail: kumarkmbios@gmail.com

1.INTRODUCTION

Sheeppoxvirus (SPV), goatpoxvirus (GPV) and lumpy skin disease virus (LSDV) are the genus of capripox virus belongs to the Poxviridae family and can cause sheeppox, Goatpox, and Lymphy Skin Diseases in sheep, goat and cattle respectively [1]. They are double stranded DNA enveloped viruses which causes significant economic losses by affecting domestic animals with substantial impact on the livelihoods of small-scale farmers in endemic regions up to 80% of high morbidity and 5-50% of mortality, but also because of significant economic losses of milk, meat and wool production [2][3][4]. Transmission of capripox is efficient and suspected to occur via aerosol and insect vector [5]. The clinical symptoms are classic vesicular form was usually observed and characterized by the appearance of skin lesions on the entire body surface evolving from macules, papules, vesicles or vesiculo- pustules and crusts at the end of disease evolution[6].

Capripox diseases are notifiable to Office International des Epizooties and enzootic in different country parts like Africa, Middle East and Asia including Indian sub-continent along with into Europe from Middle-East regions as transportation of infected animals[7][8]. Recently through import of live cattle that carries the LSDV from endemic countries has been aggressively spreading in the Europe, India, south-west of Middle East and other countries

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[9]. Lymphy Skin Disease was first reported in 1929 in south of Africa in cattle and then spread in most of the central and northern of African countries [10]. Generally, capripoxvirus infections are host specific in nature and occasionally infect more than one species [11].

The aim of the study was Capripoxviruses have the threats to become potential emerging disease because of global climatic change and due to porous international boundaries which facilitate for migration of animals and animal products from neighbouring countries. A28 is the membrane component of the intracellular mature virion, during the virus replication cycle the poxvirus A28 protein is expressed at late times and it is suggesting that all poxviruses use a common A28-dependent mechanism of cell penetration [12].The analysis of fussion protein along with vaccnia virus and their structural Prediction and physiochemical parameters assessment is stable and this structural information of this model can be effectively used and can be further implemented in future diagnosis,vaccine and drug designing.

2. MATERIALS AND METHODS

2.1. Sequence retrieval: The *Capripox* viruses membrane component of the intracellular mature virion is the fussion envelop protein A28 sequences were retrieved from UniPort. UniProt is the protein database to provide the scientific community with a comprehensive, high-quality,functional information and freely accessible resource of protein sequence (www.uniprot.org). The accession numbers are P16718 is the Fusion envelope protein Sheeppox virus, A0A2Z4XFP4 envelope protein of Goatpox virus, Q8JTR0 fusion of Lumpy skin disease virus and P68633 Vaccinia virus.

2.2. Physico-chemical and secondary structural characterization: The Physico-chemical characterization of Capripox viral A28 fusion protein was carriedout along with Vaccinia virus A28 envelop fusion protein by using Expasy's ProtParam server (http://us.expasy.org/tools/protparam.html) [13][14][15]. The results were showed in Table 1.

2.3. Homology modeling and structural validation: Homology modeling and 3D structural prediction of Capripox vial envelop p32 proteins was carried out by using phyre2, (http://www.sbg.bio.ic.ac.uk/phyre2)which uses advanced remote homology detection methods to build 3D models. The evaluation of protein structural models was performed by using PROCHECK, Swiss-PdbViewer software and Finally chosen model was visualized by Rasmol tools[16][17][18][19].

2.4. Smart domain analysis: SMART is a Webtool (Simple Modular Architecture Research Tool), as well a growing collection of profiles for shuffled extracellular domains and it allows users to quickly identification and annotation of protein domains and the exploration of protein domain architectures **(http://smart.embl.de/)**[20] [21].

3. RESULTS AND DISCUSSION

3.1. Retrival of Genome sequences: The sequence are retrived from UniPort in FASTA format for furthere analysis and structural predictions.

>A28_SHEV_Envelopeprotein_P16718
MNAITIFFIILSTVAVCIIIFQLYSIYLNYDNIKEFNSAHSAFEFSKSVNTLSLDRTIKDPN
DDIYDPKQKWRCVKLDNDYVSVSMFGFKSNGSEIRKFKNLESCIDYTFSQSTHSDIK
NPCILQNGIKSKECIFLKSMF
>A28_LSDVN_Envelopeprotein_Q8JTR0
MNAITIFFIILSTVAVCIIIFQLYSIYLNYDNIKEFNSAHSAFEFSKSVNTLSLDRTIKDPN
DDIYDPKQKWRCVKLDNDYVSVSMFGFKSNGSEIRKFKNLESCIDYTFSQSTHSDIK
NPCILQNGIKSKECIFLKSMF

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>A28_A0A2Z4XFP4_GPV
MNAITIFFIILSTVAVCIIIFQLYSIYLNYDNIKEFNSAHSAFEFSKSVNTLSLDRTIKDPN
DDIYDPKQKWRCVKLDNDYVSVSMFGFKSNGSEIRKFKNLESCIDYTFSQSTHSDIK
NPCILQNGIKSKECIFLKSMF
>A28_VACCW_P68633_Envelope protein
MNSLSIFFIVVATAAVCLLFIQGYSIYENYGNIKEFNATHAAFEYSKSIGGTPALDRRV
QDVNDTISDVKQKWRCVVYPGNGFVSASIFGFQAEVGPNNTRSIRKFNTMQQCIDFT
FSDVININIYNPCVVPNINNAECQFLKSVL

3.2. Physico-chemical and secondary structural characterization:

The computational analysis of physicochemical and secondary structural properties of Intracellular Mature Virion A28 proteins of capripox virus along with vaccinia viral virion A28 was done. Among all four viruses sheeppox, goatpox, and lymphyskin disease shown similar properties except vaccinia virus, the detailed results were presented in Table 1& 2.

Table. 1 Physico-chemical Parameters computed using Expasy's ProtParam tool

Property	A28 Envelop Fusion Protein			
	Sheeppox virus	**Goatpox virus**	**Lumpy skin disease virus**	**Vaccinia virus**
Number of amino acides	140	140	140	146
Molecular weight	16202.63	16202.63	16202.63	16328.61
Theoretical pl	7.59	7.59	7.59	6.54
Total number of negatively charged residues(Asp+Glu)	15	15	15	11
Total number of positively residues (Arg+Lys)	16	16	16	11
Extinction coefficient (0.907, assuming all pairs of Cys residues form cystines)	14690	14690	14690	14690
Extinction coefficient* (0.891, assuming all Cys residues are reduced)	14440	14440	14440	14440
Instability index	25.83	25.83	25.83	28.13
Aliphatic index	87.71	87.71	87.71	88.08
Grand average of hdropathicity	-0.094	-0.094	-0.094	0.098

Table. 2 Secondary structure elements values by SOPMA.

Property	Value(%) of A28 major envelope protein			
	Sheeppox virus	**Goatpox virus**	**Lumpy skin disease virus**	**Vaccinia virus**
Alpha helix (Hh)	46.43%	46.43%	46.43%	41.78%
310 helix (Gg)	0.00%	0.00%	0.00%	0.00%
Pi helix (Ii)	0.00%	0.00%	0.00%	0.00%
Beta bridge (Bb)	0.00%	0.00%	0.00%	0.00%
Extended strand (Ee)	21.43%	21.43%	21.43%	22.60%

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Beta turn (Tt)	4.29%	4.29%	4.29%	5.48%
Bend region (Ss)	0.00%	0.00%	0.00%	0.00%
Random coil (Cc)	27.86%	27.86%	27.86%	30.14%
Ambiguous states (?)	0.00%	0.00%	0.00%	0.00%
Other states	0.00%	0.00%	0.00%	0.00%

3.3 structural prediction and validation

structural prediction was constructed using Phyre2 homology model. Phyre2 is a online server which is used to predict and analyze protein structure, function and mutations. In this study the FASTA sequence of mature virion protein A28 was used to attain 3D models.The modelled and validated protein checked by PROCHECK the results were shown figure **1. A,** 1B of Sheeppox virus, 2.A, 2B of Goatpox virus, 3.A,3B Lymphy Skindisease virus and4. A, 4B of Vaccinia Viruses [22].

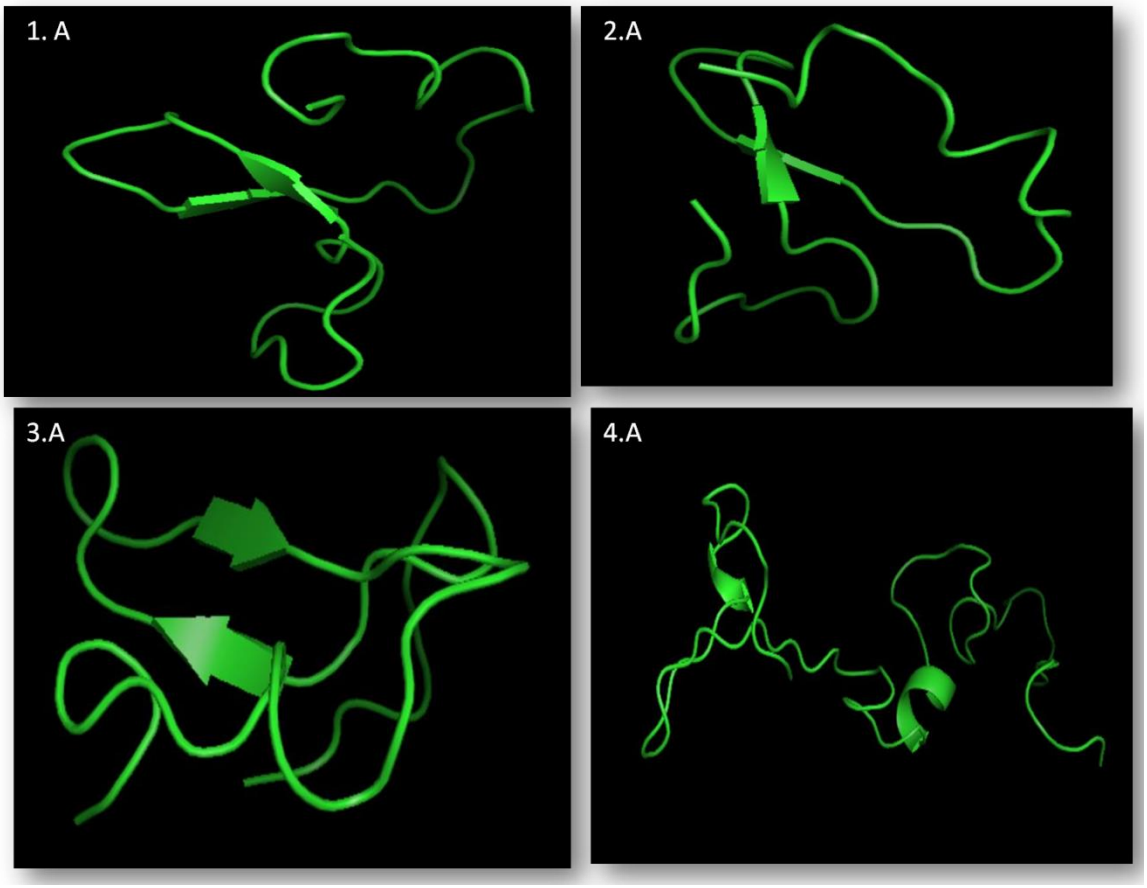

Figure 1. Modelled Fusion protein A28 shown. 1. A. Sheeppox virus, 2. A. Goatpox virus, 3.A. Lymphy Skindisease virus, 4. A. Vaccinia Virus.

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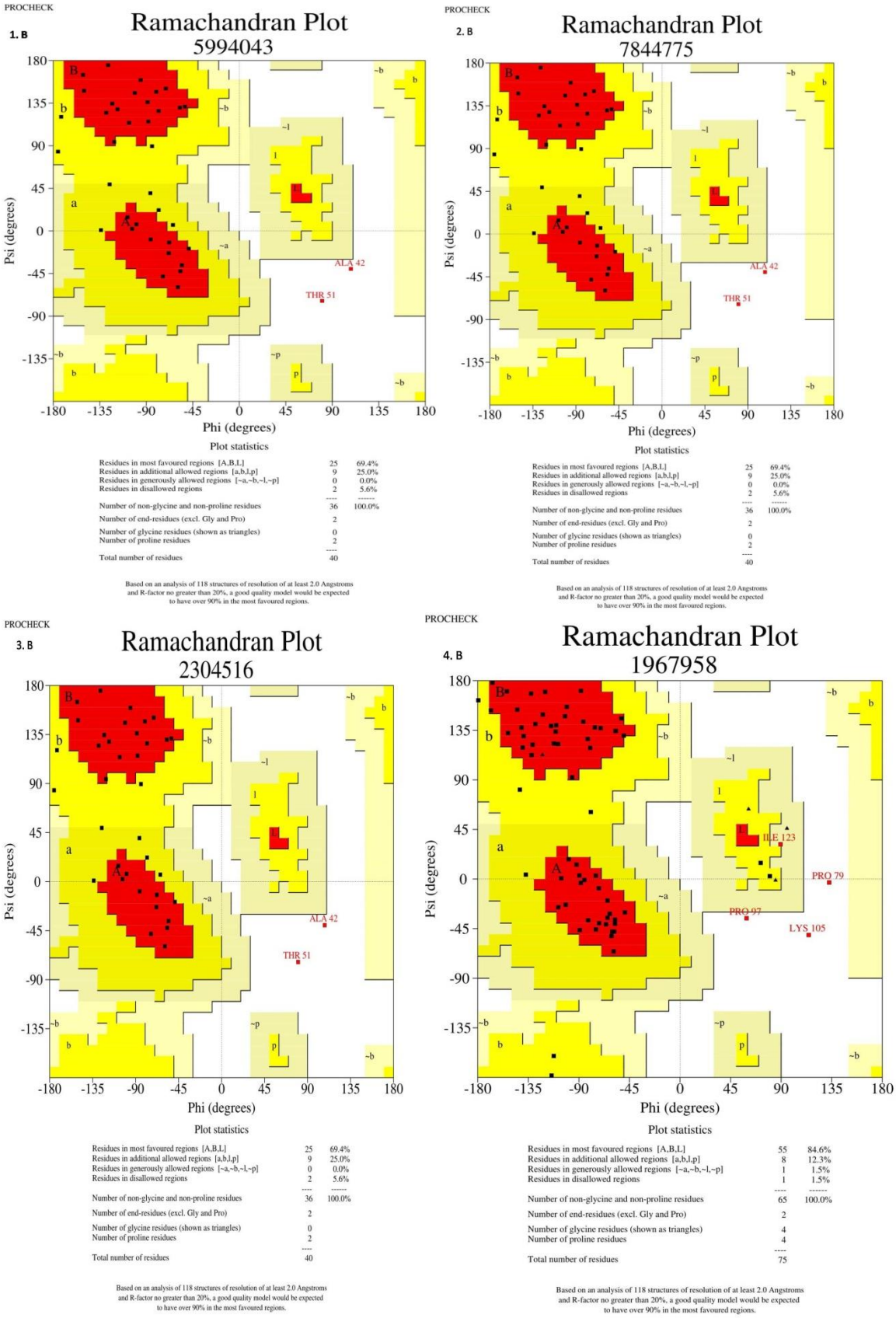

Figure 2. Ramachandran Plot. 1. B. Sheeppox virus, 2.B. Goatpox virus, 3.B. Lymphy Skindisease virus, 4. B. Vaccinia Virus.

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3.4 SMART domain Analysis. The Capripox viral intracellular mature virion A28 structural domain identification was performed using the SMART (Simple Modular Architecture Research Tool) online tool (http://smart.embl-heidelberg.) the result were shown below the table (Table 3 and 4). The outlier homologues and homologues of known structure A28 shown one scop domain was charactise of Structural Classification of Proteins SCOP:d1a8i of SHPV, LSDV of SCOP:d1a8i, GPV SCOP:d1a8i were shown similar properties where as in vaccinia virus shows two scope domains such as SCOP:d1div_2 and SCOP:d1epxa represents Superfamily: Aldolase.

Table. 3 Confidently predicted domains, repeats, motifs and features:

Name	Start	End	E-value
SHPV_Pfam:Pox_A28	22	161	7.8E-63
LSDV_Pfam:Pox_A28	1	140	4.1E-66
GPV_Pfam:Pox_A28	1	140	4.1E-66
VCCV_Pfam:Pox_A28	1	146	6.7E-67

Table. 4 Outlier homologues and homologues of known structure:

Name	SCOP domain	Sequence	Start	End	E-value
SHPV_Pfam:Pox_A28	SCOP:d1a8i__	SAHSAFEFSKSVNTLSLDR TIKDPNDDIYDPKQKWR	58	94	4.5
LSDV_Pfam:Pox_A28	SCOP:d1a8i__	SAHSAFEFSKSVNTLSLDR TIKDPNDDIYDPKQKWR	37	73	3.2
GPV_Pfam:Pox_A28	SCOP:d1a8i__	SAHSAFEFSKSVNTLSLDR TIKDPNDDIYDPKQKWR	37	73	3.2
VCCV_Pfam:Pox_A28	SCOP:d1div_2	FIQGYSIYENYGNIKEFNA THAAFE	19	44	0.26
	SCOP:d1epxa_	WRCVVYPGNGFVSASIFG FQAEV	72	95	9.6

4. CONCLUSION

In this work, we predicted the 3D structures and SMART domain identification for membrane component of the intracellular mature virion A28 of Sheeppox, Goatpox, Lymphy Skin Disease virus and Vaccinia virus. by various informatics tools and servers. Knowing the structure, physicochemical properties and functional structural domains of protein is of great importance for understanding the molecular mechanisms of these proteins during their evolutionary status with emerging poxviruses. As well this model can be effectively used and can be further implemented in future diagnosis, vaccine and drug designing.

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COMPARATIVE ANALYSIS OF ANTIFUNGAL ACTIVITY OF NATURAL PLANT EXTRACTS VERSUS KETOCONAZOLE AND SALICYLIC ACID AGAINST MALASSEZIA

Sharma Chahat, Tiwari Vrinda and Florence Shoba*
School of Sciences, Department of Life Sciences, Garden City University, Bengaluru 560049

ABSTRACT: Dandruff is the major problem for many people in today's world. It is a chronic scalp condition characterized by scaling, itching and redness of the scalp. Many anti-dandruff shampoos, whether synthetic or herbal, and lotions are marketed to combat this problem. The present study was done with an intention of finding the antifungal activity of plant extracts on *Malassezia* the fungi which causes dandruff and also comparing its activity with that of ketaconazole and salicylic acid the commonly used antifungal agents. *Malassezia* was isolated from dandruff samples by using a direct placement method. The antifungal activity of five plants: *Aloe vera, Ocimum basilicum, Azadirachita indica, Murraya koenigii and Linum usitatissimum* was tested by using agar diffusion method against the Malassezia isolated. Ketaconazole and salicylic acid were also tested against the same. The antifungal activity was observed in the form of zones of inhibition. Different concentrations of plant extracts were taken to check the Minimum Inhibitory Concentration (MIC). All the selected plants were proved to be effective as they all showed inhibition against *Malassezia*. Ketaconozole and salicylic acid were proved to be more effective compared to plant extracts.Amongst the plant extracts the highest zone of inhibition was shown by *Aloe vera* while minimum inhibition was observed by Neem. *Aloe vera* and flax showed maximum antifungal activity. These results confirm the antifungal nature of plant extracts and support the traditional use of plant therapy on the fungal infection dandruff caused by *Malassezia spp*.

Keywords: Dandruff,*Malassezia*, Ketaconozole, Salicylic acid, *Aloe Vera*, Neem.

***Corresponding Author:** Ms. Florence Shoba. Dept. of Life Sciences, Garden City University, Bangalore, Karnataka, India. Email Address: florence.shoba@gardencity.university

1. INTRODUCTION

Dandruff is the major problem for many people living in any part of the world. Dandruff is a common condition which is generally caused due to hormonal changes and fluctuations, genetic, pollution, environmental changes and personal hygienic conditions.Dandruff is a skin condition that mainly affects the scalp. Symptoms include itchy scalp, flakiness with red and greasy patches on the skin, one can get it behind the ears, eyebrows, or even in folds of the face. It can be red, flaky, itchy, or a combination of all those factors, which is a more severe form of the condition, which includes inflammation of the skin, is known as seborrhoeic dermatitis. Another type of dandruff called cradle cap can affect babies. This disorder, which causes a scaly, crusty scalp, is most common in newborns, but it can occur anytime during infancy. [10]

According to the symptoms dandruff is classified into two types – Dry (common) and Oily. Dry dandruff also known as *Pityriasis simplex* is characterized by excessive formation of minute scales of white grayish or ashen color, accumulating on the scalp area. These scales are at first localized in the middle of scalp area and then spread towards parietal, frontal and occipital areas. In this type of dandruff, no excessive hair loss is observed. The other type of

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dandruff is called oily dandruff or *Pityriasis steatoides*. It arises on the scalp skin with varied intensity of sebum production. Inflammation of varied intensity develops on the scalp skin along with the appearance of oily scales of dirty yellow color that can form lesions. Hair fall is common; it may also exacerbate and rogenetic alopecia. The most common site affected by this type of dandruff is scalp, but it can occur between eyebrows, along the side of nose, behind the ears, over the breastbone and sometimes in the armpits. [7]

Dandruff scale is a cluster of corneocytes, which have retained a large degree of cohesion with one another and detach as such from the surface of the stratum corneum.

Dandruff can almost be controlled and effectively treated, but the treatment of dandruff may take a little patience and persistence. In general, daily cleansing with a gentle shampoo to reduce oiliness and skin cell buildup can often help mild dandruff. When regular shampoos are not effective, dandruff shampoos can be used. Also, dandruff shampoos are not all alike, and one may need to experiment until they find the one which best suits them. The formulations must be suitable for hairy regions and combat the dandruff conditions. It is therefore essential that these formulations have accepted pharmaceutical properties at the cosmetological level. Different types of formulations like shampoos, creams, lotions, emulsions, hair oils and other cosmetic formulations are readily available in the market that are used to control dandruff. These formulations include therapeutic use of anti-dandruff agents that are classified into three groups according to their mechanism of action;

- Fungicidal substances: e.g., zinc pyrithione and imidazoles.
- Cytostatic substances: e.g., tar, selenium sulfide and octopirox.
- Keratolytic substances: e.g., salicyclic acid and sulfur compounds, ketconozole. [1]

The most common cause of dandruff is the metabolic by-products of skin micro-organisms most specifically Malassezia yeasts. *Malassezia* (formerly known as *Pityrosporum*) is amonophyletic and unipolar lipophilic yeast. It is naturally found on the skin surfaces of many animals, including humans and associated with a variety of conditions including dandruff, atopic eczema/dermatitis, pityriasis versicolor, seborrheic dermatitis and folliculitis (Ashbee and Scheynius, 2010) [2]. It is a part of natural body flora. It lives quietly and unnoticed on our body, usually without our being aware of its presence unless stress, illness, antibiotics or other unfavorable conditions upset the natural balance of our body's immune response(Gueho *et al*., 1996) [6]. Currently there are at least 14 recognized species of Malassezia ie. *M. caprae*, *M. cuniculi*, *M. dermatis*, *M. equina*, *M. furfur*, *M. globosa*, *M. japonica*, *M. nana*, *M. obtusa*, *M. pachydermatis*, *M. restricta*, *M. slooffiae*, *M. sympodialis* and *M. yamatoensis*; out of which *M. furfur* is restricted to the human host, *M. sympodialis* is a cat and human pathogen and *M. pachydermatis*, the causative agent of canine otitis externa is also a human pathogen.[3]

An antidandruff drug may be described as a cosmetic preparation required for the hair and scalp, packed in a form which is convenient for use. Antidandruff drugs and plant extract are used against this dandruff causing fungi. Two types of shampoos are used:[9]

1. Synthetic anti-dandruff shampoos (based on ingredients of chemical origin) which mainly contain salicylic acid and ketaconozole.
2. Herbal anti-dandruff shampoos (based on plant ingredients) or natural plant extracts are directly used for the treatment of dandruff.[11]

Plants represent a rich source of antimicrobial agents. Plants are used medicinally in still around the world different countries and are a source of many potent and powerful drugs. Throughout history, medicinal plants have been paramount in the treatment of disease, for people – from the commoners to those in power. Medicinal plants have been used to treat illness and disease for thousands of years ago. Even now they are economically important, being used in pharmaceutical, cosmetic, perfumery, and food industries. A wide range of

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medicinal plant parts are used for extractions as raw drugs and they possess varied medicinal properties. The different parts used include root, stem, flower, fruit, twigs exudates and modified plant. In recent years we've seen an explosion in the number of natural products and beauty products containing natural extracts. At this point only around 10% of the vast range of plant species has been discovered by man, meaning the potential for many more effective cures is there, just waiting to be discovered and utilized. [12]

A wide range of antifungals such as ketoconazole, zinc pyrithione, salicylic acid, imidazole, ciclopirox and selenium disulfide have been found to be effective in the treatment of dandruff, ie. found effective in inhibiting the growth of the organism. These antifungals constitute the major components of the anti-dandruff shampoos. Although these antifungals are very much effective in the treatment against Malassezia, but it is seen that on stopping the treatment the dandruff seems to return back again. Therefore, people have started looking for ayurvedic treatments. As Ayurveda has known to be the oldest healing science and has the remedy for every possible health ailments without any side effects. Ayurveda suggests many natural plants such as coconut, lemon, mint, fenugreek, amala, neem, aloe vera and shikakayee.

2. MATERIALS AND METHODS

2.1 Isolation of *Malassezia*

Sample Collection: Flakes or scales were collectedfrom scalp by partitioning the hair with a sterile comb and scrapping approximately one inch area using a sterile blunt scalpel. The specimen was then transferred into a dark sampling paper to prevent exposure to sunlight. The samples were inoculated on Sabouraud Dextrose Agar (SDA) by direct placement method.The plates were then incubated at 30 °C for 7 days, which were observed regularly.

2.2 Characterization of Malessezia

2.2.1 Direct microscopy: A drop of 10% KOH was addedonto a clean slide containing the smear of sample and covered using a coverslip. The sample was then heated over a Bunsen burner to remove bubbles. The slides were viewed under 40X objective lens. [4,5,6]

2.2.2 Biochemical Tests :

Catalase Test: Catalase test was carried out to ascertain thepresence of *Malassezia* species as it is catalase positive. Few drops of 3% hydrogen peroxide (H2O2) solution were added into a petri plate/ glass slide. Several colonies of the isolated fungal colonies were immersed into the glass slide using a sterile inoculation loop and observed for effervescence.

Esculin Hydrolysis Test: The medium used was bileesculin agar which is a nutrient agar-based medium containing 0.1% esculin and 10% bile salts. The bile salt inhibits some bacteria, and also shows the ability to grow in the presence of bile salts represents a second test use for the medium. Pure culture of Malassezia was aseptically aseptically inoculated into esculin agar and incubated at 30 °C for 24 h and the result was observed.

2.3 Plant sample collection

Five different plants *Aloe vera, Ocimum basilicum, Azadirachita indica, Murraya koenigii and Linum usitatissimum* were collected from different areas of K.R Puram, Bangalore.Their soxhlet extracts were used to determine their antifungal activity against Malassezia.Their antifungal was compared with that of Ketaconazole and Salicylic acid which are two of the common antifungal agents used in the treatment of dandruff.

2.4 Antifungal activity:

2.4.1 Agar diffusion Method: Agar diffusion method was performed to check the antifungal activities of ketaconozole, salicylic acid and natural plant extract of *Aloe vera, Ocimum*

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basilicum, Azadirachita indica, Murraya koenigii and Linum usitatissimum. A soxhlet extract of the five plants was done using methanol.L N media was used to prepare plates. Two days prior inoculated culture of *Malassezia* species in L N broth was maintained to be used for this assay. 500 µL of culture suspension was spread on the petri plates. Wells of the size 0.6 cm in diameter were bored using a cork borer in which 100 µL of 100% concentrations of different plant extracts, ketaconazole and salicylic acid was added using micro-pipette. Experiments were done in duplicates with suitable controls. After incubation, the plates were observed. The inhibition zone was measured using a zone measuring scale and results were recorded.

2.4.2 Minimum Inhibitory Concentration (MIC): MIC was performed in L N agar plates by agar diffusion method. 24 h active culture of the test organisms were used for this study. The culture of *Malassezia* in L N broth was used for inoculation and incubated at 30°C for 24 h. The same protocol was followed as mentioned above. The concentrations to check the MIC for a given sample used were 10%, 30%, 50%, and 100% (v/v). The dilutions were done using sterile distilled water. Experiments were performed in duplicates with suitable controls.

3. RESULTS AND DISCUSSION

Malassezia was isolated from the dandruff samples and confirmed by direct microscopy and biochemical tests. It was found positive for catalase and esculin hydrolysis.

3.1 Anti-fungal Activity

All the five plants: *Aloe vera, Ocimum basilicum, Azadirachita indica, Murraya koenigii and Linum usitatissimum* showed antifungal activity against Malassezia, the zones of inhibition were as tabulated.

Name of plants	Diameter of Zone of Inhibition (in mm)
Azadirachta indica	1mm
Murraya koenigii	2mm
Aloe vera	1mm
Ocimum basilicum	2mm
Linum useitatissium	3mm

Table 1: Antifungal activity of methanolic plant extracts against Malassezia

Antifungal drugs	Zone of inhibition (in mm)
Ketoconozole	10mm
Salicylic acid	8mm

Table 2: Antifungal activity of Ketoconozole and Salicylic acid against Malassezia

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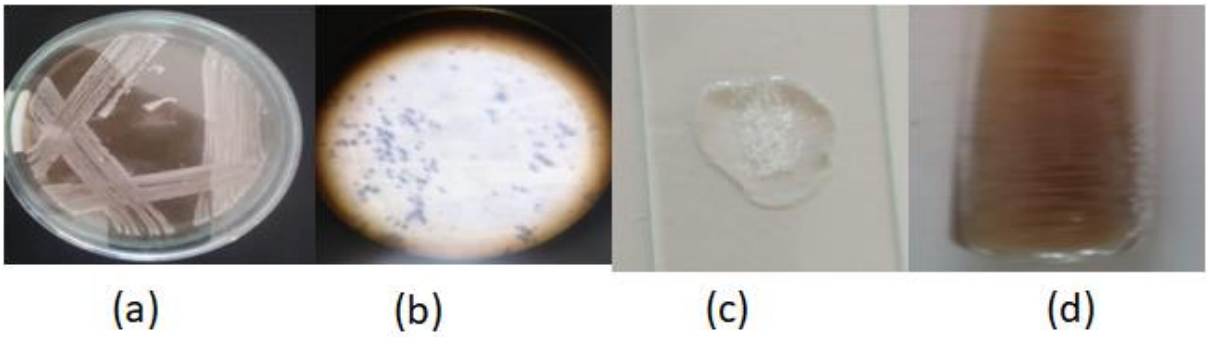

Figure 1: Isolation of *Malassezia*; (a) Pure culture *Malassezia*; (b) Direct microscopy using KOHshowing ;(c) Positive Catalase test; (d) Positive esculin hydrolysis

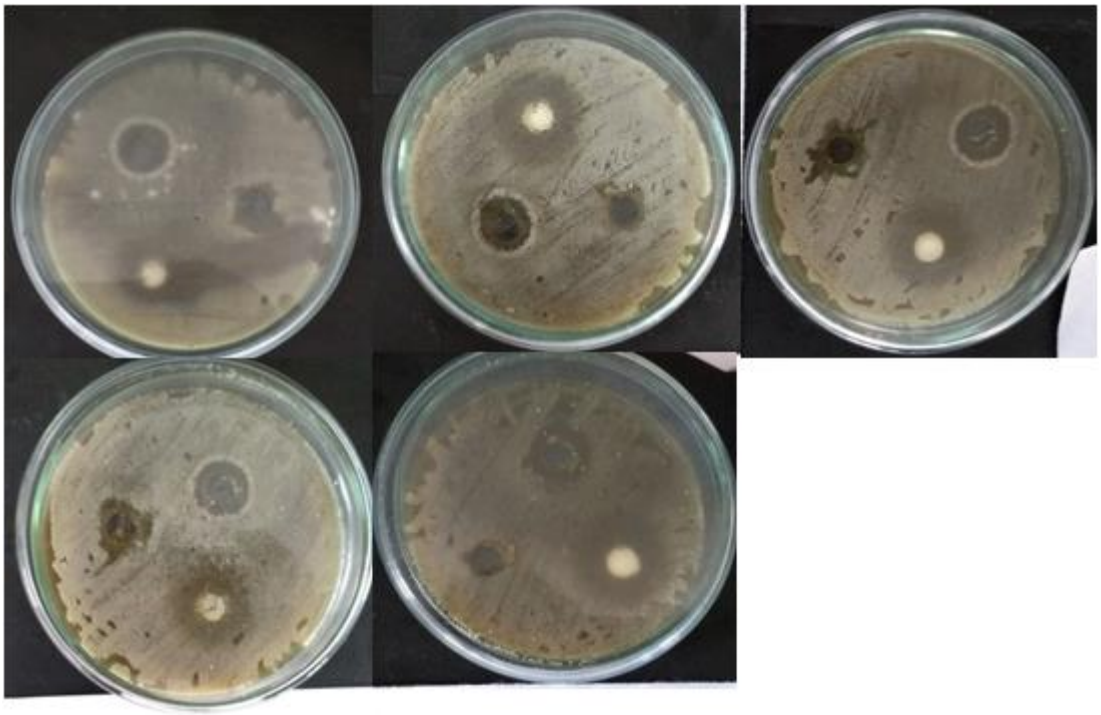

Figure 2: Antifungal activity of methanolic extracts of plants against *Malassezia* versus ketoconazole and salicylic acid.

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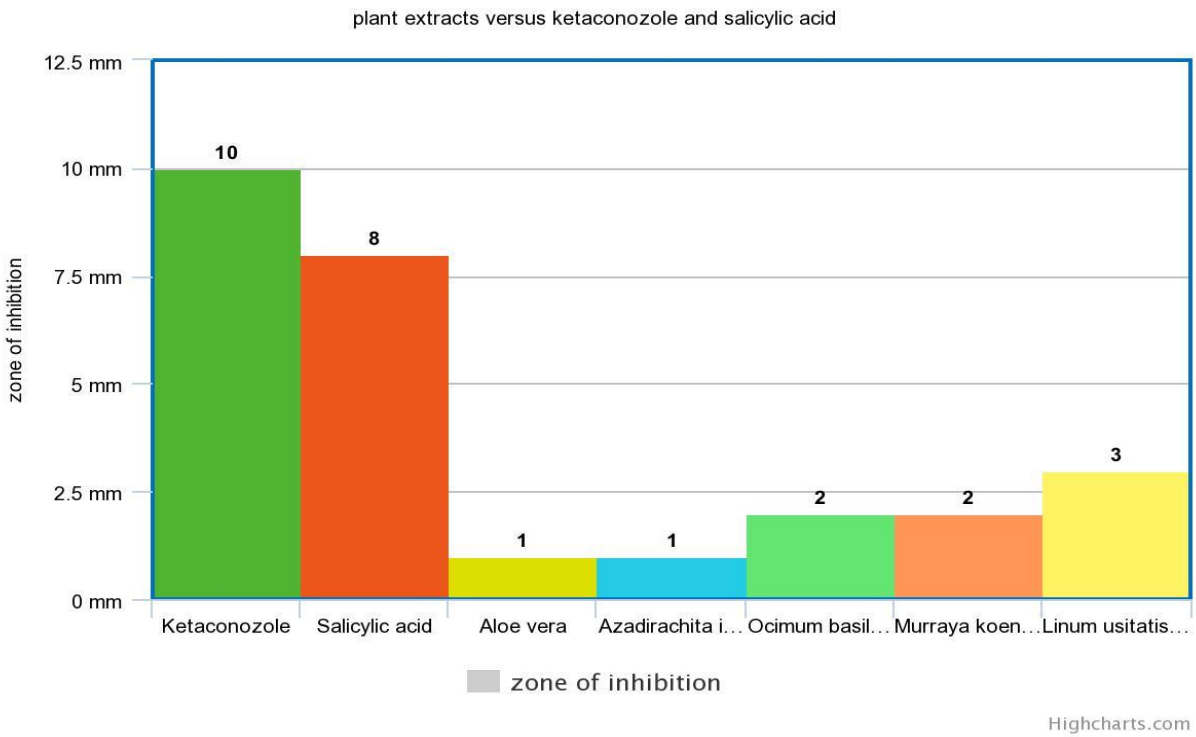

Figure 3: Comparative analysis of zone of inhibitions of 100% methanolic plant extracts, versus ketoconazole and salicylic acid.

3.2 Minimum Inhibitory Concentration (MIC): TheMIC was performed using different concentrations various plant extracts, ketoconazole and salicylic acid ie. 10mg/ml, 30mg/ml, 50mg/ml and 100mg/ml. So that the minimum inhibitory concentration of plant extracts, ketoconazole and salicylic acid can be determined which proves the anti-fungal activity of the samples even at very less concentrations. The results were as tabulated..

Samples	**10mg/ml**	**30mg/ml**	**50mg/ml**	**100mg/ml**
Ketoconozole	6mm	9mm	10mm	15m
Salicylic acid	8mm	12mm	14mm	17mm
Aloe vera	12mm	16mm	19mm	22mm
Ocimum basilicum	7mm	9mm	10mm	12mm
Azadirachta indica	No zone	No zone	3mm	8mm
Murraya koenigii	No zone	No zone	No zone	No zone

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Linum usitatissimum	7mm	9mm	12mm	14mm

Table 3: Minimum inhibitory concentration of methanolic extracts of different plant extracts, ketoconozole and salicylic acid

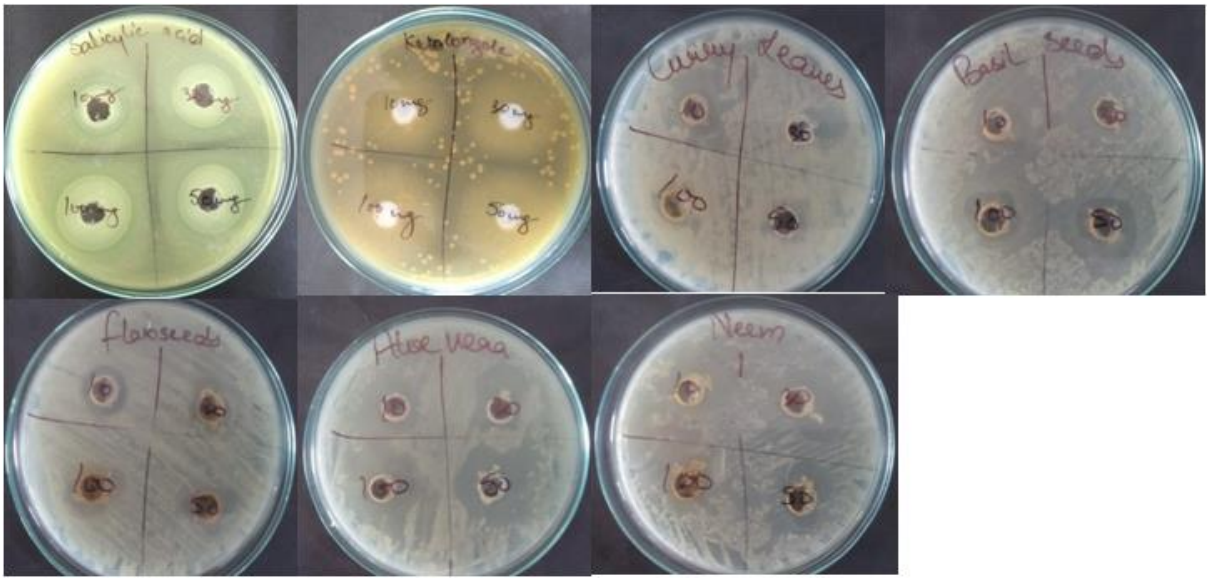

Figure 4: MIC of different plant extracts, ketoconazole and salicylic acid

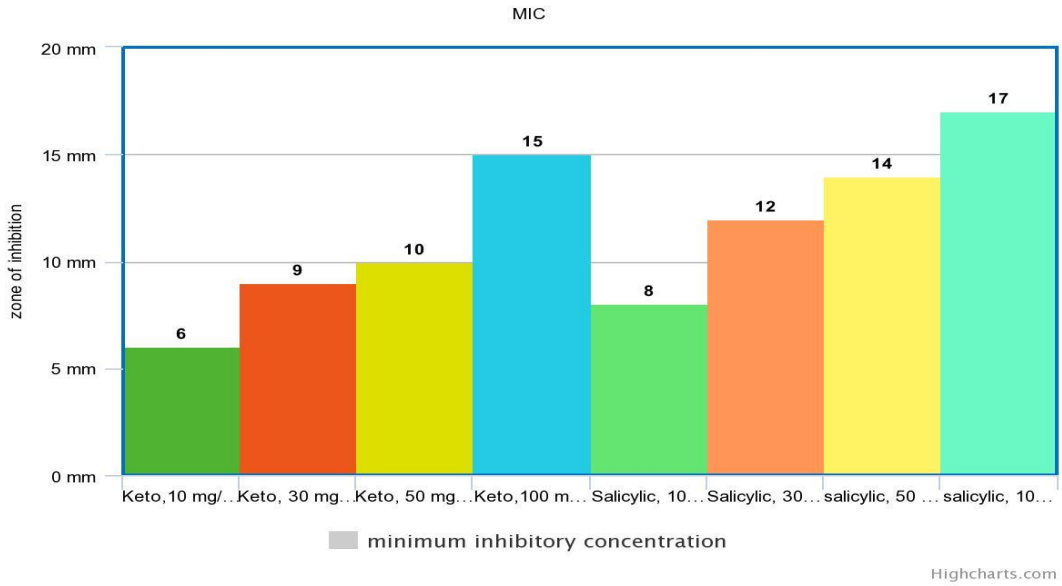

Figure 5: MIC of Ketoconazole and Salicylic acid

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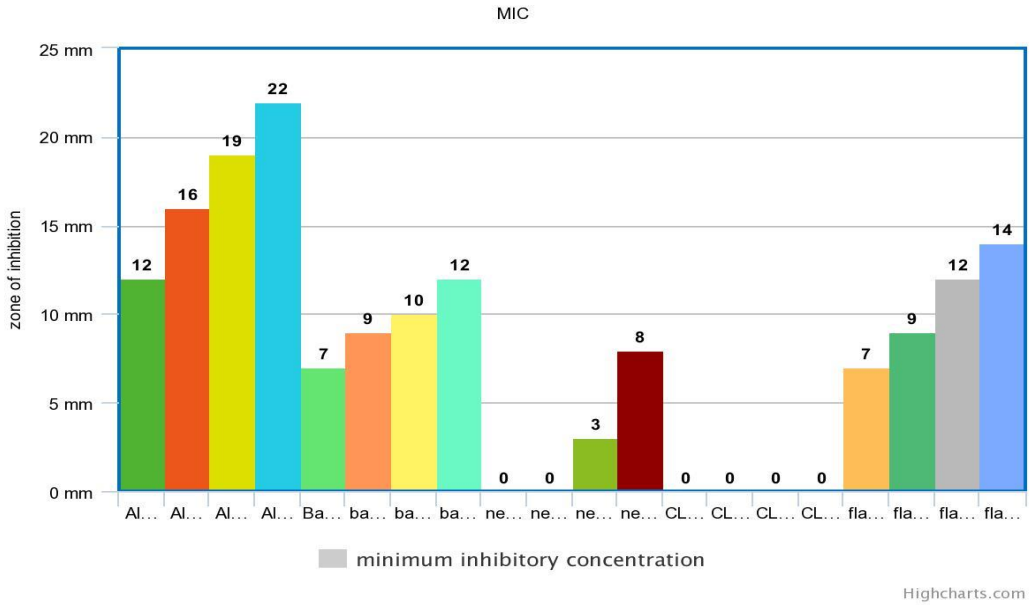

Figure 6: MIC of different plant extracts

4. CONCLUSION

The study was to determine carry out comparative analysis of antifungal activity of natural plant extracts versus ketoconazole and salicylic acid. Ketoconazole and salicylic acid are widely used antifungals available in market for dandruff related problems which is caused by Malassezia spp., plant extracts of plants such *as Aloe vera, Ocimum basilicum, Azadirachta indica, Murraya koenigii* and *Linum usitatissimum* showed antifungal activity against the dandruff causing fungi. *Murraya koenigii, Ocimum basilicum* and *Linum usitatissimum* showed maximum antifungal activity. Even *Aloe vera* showed great antifungal activity against Malassezia spp. at very low concentrations ie. 10mg/ml. Also, the use of basil seeds and flax seeds showing antifungal activity against the Malassezia spp. has never been performed earlier. The zones of inhibition of *Aloe vera, Ocimum basilicum* and *Linumusitatissimum* were very close to that of ketaconazole and salicylic acid which indicates the potential of these plants in the treatment of dandruff caused by Malassezia.The zone of inhibition of *Aloe vera* is infact greater than that shown by bothe kataconazole and salicylic acid.These results confirm the antifungal nature of plant extracts and support the traditional use of plant therapy of fungal infections especially dandruff caused by Malassezia spp. concern has been expressed about rising prevalence of pathogenic microorganisms which may become resistant to antifungals available in market. Also, there is a problem posed by high cost, adulteration and increasing toxic side effects of the synthetic drugs coupled with their inadequacy in disease treatment. These plants possess antifungal properties against Malassezia spp. and infections caused by them. Isolated methanolic extracts of these plants can serve as therapeutic agents and thus can be used as potential bioactive compounds for treating infections caused by Malassezia spp.

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ISOLATION AND CHARATERIZATION OF PHA PRODUCERING BACTERIA USING SUGAR MILL EFFLUENT

Shruti Awasthi [1]*, Kalyani Kamat [2] and Yasrib Qurishi [3]
[1]Department of Life Sciences, Garden City University, Bangalore
[2]Department of Microbiology, Garden City University, Bangalore
[3]Department of Life Sciences, Garden City University, Bangalore

ABSTRACT: Polyhydroxy alkaonates are biodegradable polyesters produced by bacteria as energy and carbon reserves. These can be effectively used as bioplastic and have wide range of applications in different fields such as medicals, cosmetic industry, due to their desired features, mainly biodegradable and biocompatible nature. In this study total 20 isolates were isolated from 3 different samples and screened for PHA production. PHA production was shown by 12 isolates, out of which 6 isolates showed more intense color. These 6 isolates were subjected to nile blue assay. Isolate 1and 5 gave intense bright orange fluorescence under UV, but intensity for isolate1 was comparatively higher. Isolate1 was later identified as Pseudomonas sps. Sugar industry waste water was directly used as production media and yield obtained was 0.98g/l with total accumulation of 37.46%. Optimization study revealed that optimum initial pH, Temperature and time of incubation for PHA production by isolate 1 was 7 (in comparison to5,6,8), 35^0c (in comparison to 25^0c, 30^0c, 40^0c) and 72 hours(compared to 24, 46,96 hrs). Also, PHA- starch blend sheet was prepared by mixing PHA and starch in ratio 4:1.

Keywords: Polyhydroxy alkaonates, Bioplastic, Polysters, Cosmetic industry, Biodegradable and biocompatible nature.

***Corresponding Author**: Dr. Shruti Awasthi
Department of Life Sciences, Garden City University, Bangalore, India
Email: shruti.awasthi@gardencity.university

1. INTRODUCTION

Plastics have become significant since 1940s and since then these are replacing conventional materials like glass, wood, and other constructional materials, even metals in many industrial, domestic and environmental applications [1]. These widespread applications are due to features such as low density, strength, durability and resistance to degradation **[2].** Unfortunately these petroleum based plastics accumulate in the environment for long time and remain protected from chemical or microbial attack. Therefore, these remain buried in landfills for years and take up space contributing to environmental pollution **[3].** Thus plastic waste management is a major worldwide concern [4]**.** The synthetic plastics have harmful effects on wildlife and pose threat to environment and other serene habitats. In the face of these ecological facts, production of biodegradable polymers from renewable resources is the need of the hour **[5].** Biopolymers can be used as a replacement and can help overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and include polylactic acid, starch derivatives, cellulosic polymers and Polyhydroxyalkanoates. These are often biodegradable and nontoxic. Polyhydroxyalkanoates (PHAs)provide good, non toxic, fully degradable alternative to synthetic plastic [6,7].PHAs can be completely degraded within a year by variety of microorganisms **[8].** In this paper sugar mill waste effluent is used as media for growth of PHA producers. This is not only cost effective method but can effectively save the waste disposal cost of the sugar mill.

2. MATERIALS AND METHODS

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2.1 Isolation of PHA producing bacteria

Soil sample underneath disposed sugarcane bagasse was collected from 2 areas. Waste water sample was obtained from Sanjeevani Karkhana limited at Ponda Goa.Serial dilution was carried out under aseptic conditions, dilutions upto 10^{-6} were prepared using these samples. 10^{-2}, 10^{-4}, 10^{-6} dilutions were plated on sterile nutrient agar media containing 2% glucose. The plates were then incubated at 37^0c for 48 hours [9]**.** 20 colonies were obtained. These were subjected to sudan black assay method for initial screening.

2.2 Sudan Black assay method

0.02% alcoholic solution of Sudan black B was poured onto the isolated colonies and the plates were left undisturbed for 30 minutes. Excess stain was decanted and plates rinsed by adding 100% alcohol. The colonies that are unable to incorporate Sudan Black B appear white. While the PHA producers appear bluish black [10]**.**

2.3 Screening for PHA Producing Bacteria by viable colony method

The colonies that appeared black were subjected Nile Blue staining, a more sensitive and specific test for isolation of PHA producers. Positive isolates were inoculated on carbon rich nutrient agar media.

Incubation was carried out at 37^0c for 48 hours. The colonies were subjected to methanolic solution of Nile Blue Sulphate and plates left undisturbed for 20 minutes. Plates were then observed under UV transilluminator and the colony showing highest fluorescence (isolate1) was selected for further studies.

2.4 Isolation of genomic DNA from bacteria

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl-ammonium bromide (CTAB) method. SDS 20% w/v,Chloroform: isoamyl alcohol ,Isopropanol ,Ethyl alcohol.

2.5 DNA isolation protocol

Take 1 ml bacterial culture, centrifuge at 10000 rpm 2 min. at 4^0 C. Wash the pellet with sterile distilled water. (Centrifuge at 1000rpm 20 min. at 4 C 675 µl of extraction buffer was added and incubated at 37°C for 30 min. 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. Centrifuged at 10000 rpm for 10 min at 4°C Clear solution was collected in a sterile microcentrifuge tube. Equal volumes of Chloroform: Isoamyl alcohol (24:1) was added. Centrifuged at 10000 rpm for 10 min. at 4°C .The aqueous phase was removed and taken in a sterile microcentrifuge tube. 0.6 volumes of isopropyl alcohol was added and incubated at room temperature for 1hour. Centrifuged at 10000 rpm for 10 min. Pellet was washed in 500µl of 70% ethanol. Centrifuged at 10000 rpm for 10 min at room temperature. Pellet was dried and dissolved in 20 µl sterile distilled water.

2.6 Quantification of Isolated DNA

The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

2.7 pH

Different initial pH of the medium (5.0 to 9.0) was used to check whether pH has any effect on PHA production. The initial pH of the medium was adjusted by 1N hydrochloric acid, or sodium hydroxide.

2.8 Incubation temperature

Fermentation was carried out at different temperatures i.e 25, 30, 35 and 40°C and after 72 hr of incubation, growth and PHA production were determined.

2.9 Incubation time

Incubation at 24, 48, 72, 94 hours was carried out and optimum Time determined.

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3. RESULTS

3.1 Isolation of bacteria

A wide variety of bacteria are known to accumulate PHA. Today, approximately 150 different hydroxyalkanoic acids are known to be incorporated into polyhydroxyalkanoates, with microbial species from over 90 genera being reported to accumulate these polyesters. These bacteria have been reported from various environments, but only a few from the waste water and sludge ecosystems [7]. In this study, 20 isolates obtained from 3 different samples were screened for PHA producing ability.

3.2 Sudan Black assay method

Isolates were inoculated on nutrient agar containing 2% glucose and incubation was carried out at 37^0c for 48 hours. Sudan Black solution was discarded after 30 minutes and plates washed with 98% ethanol. PHA producing colonies appeared black. While those colonies which do not produce PHA are unable to incorporate sudan Black and thus appear colourless. A total of 12 colonies showed black colour of varying intensity.

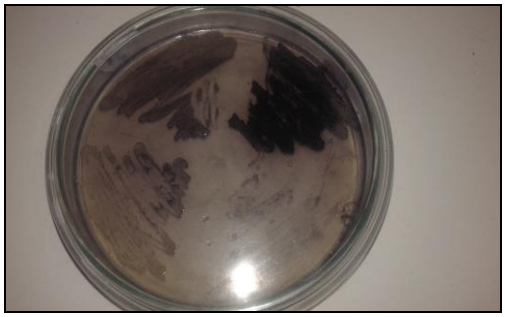

Figure 1: Sudan Balck B assay

3.3 Nile Blue Staining

6 colonies showing intense black color were selected for Nile blue assay, a more accurate and confirmatory test for PHA production. 2 colonies, isolate1 and isolate 5 showed bright orange fluorescence. But intensity of fluorescence was more for isolate 1. Isolate 1 was selected for PHA extraction.

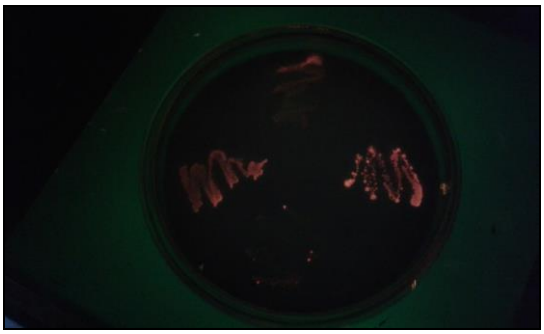

Figure 2: Nile blue staining- fluorescence shown by isolate 1 and isolate 5

3.4 PHA accumulation

PHA was accumulated at upto 0.98g/l which make up to 37.46%. Opimisation with PH, time and temperature increase the amount to 41.05 % and accumulation of 1.56g/l.

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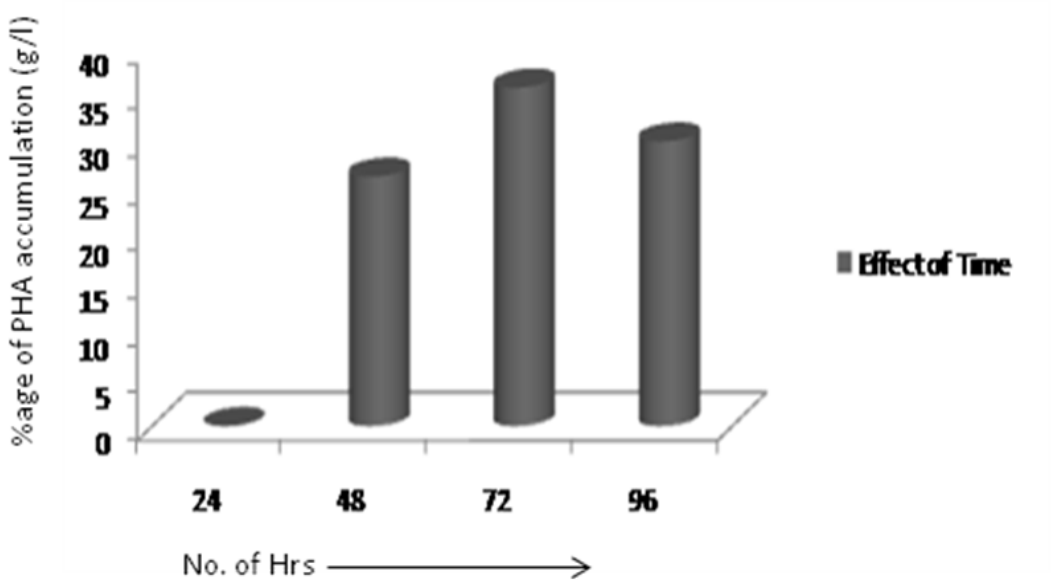

Figure 3. Effect of Time

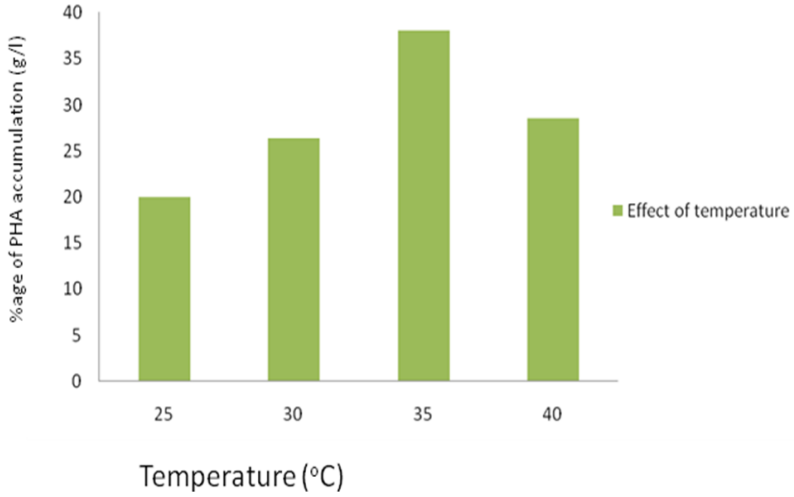

Figure 4: Effect of Temperature

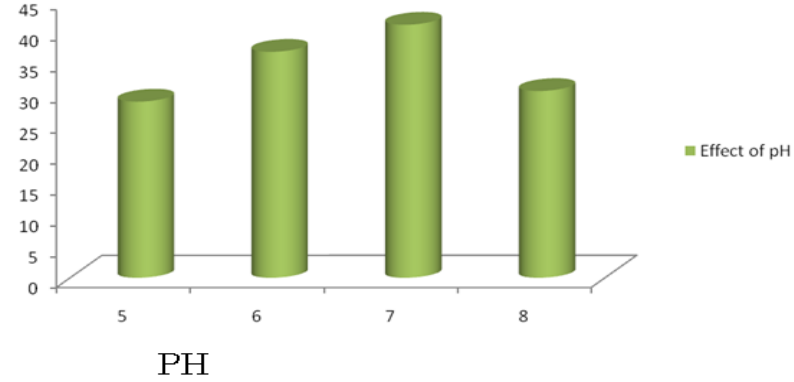

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Figure 5: Effect of PH

4. DISCUSSION

A wide variety of bacteria are known to accumulate PHA. Today, approximately 150 different hydroxyalkanoic acids are known to be incorporated into polyhydroxyalkanoates, with microbial species from over 90 genera being reported to accumulate these polyesters. These bacteria have been reported from various environments, but only a few from the waste water and sludge ecosystems[7]**.**

5. CONCLUSION

In this study 20 isolates were screened for PHA production by Sudan Black assay followed by Nile Blue assay. Isolate 1 and isolate 5 showed intense orange fluorescence under UV transilluminator. But intensity of fluorescence was more for isolate 1.In this study, sugar industry wastewater was selected as production media with objective that it will reduce costs of bioplastic production as well as waste disposal by sugar industry.Isolate accumulated 37.46% PHA which was obtained at rate of 0.98g/l. Optimum temperature, time and pH respectively were 35^0c, 72hours and 7 which allowed accumulation of 41.05%.

CONFLICT OF INTEREST None

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ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF MAJOR PHENOLIC COMPOUNDS AND DETERMINATION MINERALS AND TRACE ELEMENTS IN COMMONLY CONSUMED WHEAT VARIETIES

Mahadev C. Khetagoudar[1,3*], Madhusudhan Raddy[2], Mahadev B. Chetti[3] And V VeeraRaghvan[1]

[1]School of Bio-Chemistry, Reva University, Rukmini Knowledge Park, Kattigenahalli, Yalahanka, Bangalore - 560 064

[2]School of Chemistry, Reva University, Rukmini Knowledge Park, Kattigenahalli, Yalahanka, Bangalore - 560 064

[3]University of Agricultural Sciences, Dharwad, Karnataka -580 005

ABSTRACT: The present study evaluated major phenolic compounds and minerals and trace elements in four wheat varieties commonly consumed in India. Ultra performance liquid chromatography (UPLC) coupled with UV detector (TUV) was used for separation phenolic compounds. A unique gradient program of mobile phase composition for the separation of 22 phenolic standards have been optimized for the accurate quantification of analyte concentration in the wheat varieties. High precision was also observed in calibration slopes. The method was successfully employed to measure diverse phenolic compounds in all the four varieties of wheat. From the data it was evident that higher concentrations of various phenolic compounds were observed in all the tested varieties. Phosphorous, Na, K, Ca, Mg, Fe, Cu, Zn, Mn and S, Moisture and Ash were determined in all tested wheat varieties. There were significant differences in the minerals and trace elements between the analyzed varieties of wheat. The nutritional data suggest that the selected wheat varieties hold promise as healthy food ingredients.

KEYWORDS:UPLC-UV, Wheat, Phenolic compounds, Minerals and trace elements.

***Corrosponding Author:** Mr. Mahadev C. Khetagoudar
School of Bio-Chemistry, Reva University, Rukmini Knowledge Park, Kattigenahalli, Yalahanka, Bangalore - 560 064
E-mail Address: mahadev_arc@rediffmail.com

1. INTRODUCTION

Due to excessive use of synthetic additives peoples have more concerned about the potential health risk effects. Because of over toxicity and damage induced to DNA, the two synthetic antioxidants viz,. BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) have begun to be restricted [1]. The antioxidants isolated from plant source have gained more attraction towards the consumers safety due to ill health effects caused by synthetic antioxidants. Polyphenols are secondary metabolites, protect plants against pathogens, UV (ultraviolet) radiation and provide the cell wall integrity [2]. So for hundreds of molecules with polyphenol structure have been identified and isolated in edible plant sources. Among the plant sources fruits, vegetables, beverages, leguminous plants and cereals are very good baskets of polyphenols [3]. Scientific data proved that there is several disease preventing bioactive molecules enter the body during the consumption of whole grains [4]. Several researches proved that among the phytochemicals present in the whole cereal grains, flavonoids and phenolic acids have gained much importance because of strong antioxidant properties [5]. The most commonly found phenolic acids in cereal grains are syringic acid, vanillic acid, caffeic acid p-coumaric acid and flavonoids. Apart from these, other special class of phenolic compounds namely ferulic acid and diferulates have also been reported in

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cereal grains but these are not present in good quantities in vegetables and fruits [6]. Therefore, cereal grains consumption in large amount provides health related benefits and nutritional (calories) needs. Several literature demonstrated the phenolic compounds and their antioxidant activities on cereals, vegetables and fruits. However, a very few literature reports are available on cereal grains [7].

Wheat (*Triticum aestivum* L). is a one of the most important component of human and animal diets. During the year 2006, approximately 30% of the cereal grain consumption with greater than 600mMT consumed in the world [8]. In addition to providing calorific needs, various scientific reports had shown that diet rich with whole wheat grain can minimize the potential health risks viz., diabetes mellitus, cancer and cardiovascular diseases, providing enzymes for antioxidation and detoxification process [9]. Wheat not only provides the energy but also provides minerals and micronutrients for humans. In the field of agriculture, in order to enhance the efficiency of production the determination of minerals and trace elements is utmost important.The nutritional and calorific importance of wheat has been widely recognized as a symbol of the diets of Asian food [10]. Several survey reports on the food composition tables showed the downward trend in the food mineral contents and it may be attributed due to intensive practices in the farming system which may result in decrease of mineral contents in the soil. During the year between 1845 and the mid 1960s, the percentage and concentration of copper, magnesium, iron and zinc in wheat grains remained constant, then gradually decreased, it may be due to high-yielding and semi-dwarf cultivars introduction [11]. Several papers have hypothesized that, over the last five decades there is increased trend was observed in micronutrient malnutrition disease in global population. It may be because of evolution of high yielding varieties by modern plant breeding technique towards more agronomic yield rather than the quality of nutrition [12]. And micronutrient malnutrition is a very severe health problem in India. In the developing countries most of the children and womens are suffering from deficiency disease caused by iron, zinc and copper. Breeding techniques in wheat varieties with higher concentration of micronutrient needs skill and knowledge of the differential variation in the varieties among the available traits [13].

The phenolic compounds determination in plant materials is normally carried out in three different steps. In the first step, extraction and isolation of phenolic compounds from samples using various solvents. In the second step, clean up the extracts for the elimination of interferences. Finally, analysis and quantification of the phenolic compounds in the extracts.

All the above steps are important to get precise and accurate results. UPLC (Ultra performance liquid chromatography) with UV (Ultraviolet) detection is one of the most important versatile tool used for the both quantitative and qualitative determination of phenolic compounds in the extracts. In the modern era, columns packed with small particles gave enhanced improvement in chromatographic performance. The column packed with particle size (less than 2µm), worked at a pressure, so that higher resolution can be attained [14]. The analytical method is straight forward, reproducible polarity-independent and very rapid. In addition, no other costly chemicals and reagents are required.

In India, endemic species are grown due to the differences in climatic and geographic conditions, which contributes major important Indian diet and needs to be evaluate for phenolic composition and mineral and trace elements. A very limited knowledge about the phenolic compounds and mineral and trace elements of wheat varieties which are commonly consumed in India. The main objectives of this study were to comparative evaluation of phenolic compounds and mineral trace elements of wheat varieties commonly consumed in India. In addition, major phenolic compounds were identified and quantified by using UPLC. The results obtained by this study will provide very useful information for human health and contribute to the potential commercial application of wheat as economic natural antioxidants.

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2. MATERIALS AND METHODS

2.1 Reagents, solvents and standard phenolics

HPLC grade acetonitrile and formic acid purchased from Riedel-de Haen (Seelze Germany). Milli-Q system was used for purification of water (Bedford, MA, USA). Certified REFERENCES materials (CRM) of polyphenols (Table 1) of purity ≥ 99% were purchased from Fluka Chemie (Steinheim, Germany). Analytical grade anhydrous sodium sulphate, ethyl acetate, ethanol, vanadomolybdate, HNO_3, HCl and selenium powder were provided by Merck (India). All solvents used were filtered through 0.45µm membrane filters (Axiva, India).

2.2 Grain samples

The four Wheat (*Triticum aestivum* L.) varieties (DWR-162, UAS-316, DDK-1025 and GW-32) were procured from University of Agricultural Sciences, Dharwad, Karnataka for the present study.

2.3 Extraction of phenolic compounds

Phenolic acids were extracted by using the slight modification in the method described by Clara Fares [15]. Accurately weighed (2gms) dried and homogenized wheat samples were extracted with 70% (v/v) ethanol (50 x 2 mL) for 10h under constant stirring in room temperature. The extract was centrifuged for 20 min at 5000 rpmunder room temperature (Sigma 3K30, Germany). The supernatant was concentrated at 50°C under rotary vacuum evaporator (Hahnshin scientific co, South Korea) and using 4M HCl the pH was adjusted to 2. The phenolic compounds were separated by ethyl acetate using 50ml separating funnel (in triplicate), polled fractions were filtered under anhydrous sodium sulphate and evaporated to dryness for moisture removal. Before UPLC analysis, the phenolic acid residues was redissolved in acetonitrile (2ml) and filtered by using membrane filter (0.2µm).

2.4 UPLC methods of phenolic compounds

The Acquity UPLC (Ultra Performance Liquid Chromatography) system with TUV detector, Quaternary Gradient Pump, Sample Manager, Column oven (Waters, Milford, MA) and an Acquity UPLC™ BEH C18 column (100 mm × 2.1 mm, 1.7µm) operated at 40°C and connected with a VanGuard™ Pre-Column of the same stationary phase. Control of instruments and acquisition of data were accomplished using Empower™ 2 software. The mobile phases were (A) acetonitrile and (B) 0.1% formic acid. The gradient was linear from 0% to 30% A for 30 min, from 30% to 80% A for 5 min, and from 80% to 100% A for 5 min, followed by washing with B and reequilibration of the column for 2 min. The flow rate was 0.25 mL/min and 0.1µL was injection volume. Chromatogram were monitored on-line at 280 nm throughout the analysis.

2.5 Identification and quantification of phenolic compounds in wheat varieties

Retention time of the compounds were identified by comparing the corresponding standards peaks in the chromatogram and by spiking the samples with standards. Calibration curve was used for the calculation of each analyte concentration of the corresponding standard. All individual certified REFERENCES standards of polyphenol (1000 µg/ml) were prepared in amber colored volumetric flask by exactly weighing 10 (± 0.1) mg of each polyphenol standard and dissolving in 10 (± 0.1) ml of acetonitrile. These standard solutions were stored in freezer at -10 ºC. By diluting stock solutions the working standard mixture was prepared, from which, by serial dilution technique standards for calibration curves were prepared with acetonitrile. By Microsoft® Excel 2002 the correlation coefficient (R^2) were obtained [15].

2.6 Determination of minerals and trace elements

Procedure for determination of minerals and trace elements in the samples were prepared by

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the method as previously described with slight modification [16]. By using an atomic absorption spectrophotometer (Perkin-Elmer, MA, USA) Calcium(Ca), magnesium (Mg), copper (Cu), iron (Fe), zinc (Zn), manganese (Mn) and Sulphur contents in the extract were determined. For the estimation of calcium (Ca) and magnesium (Mg), a $LaCl_3$ solution was used. A Flame photometer (Systronics, India) with an air-propane flame was used for quantification of sodium (Na) and potassium (K) concentrations. Phosphorus was estimated by the method of vanadomolybdate using spectrophotometer (Labomed, Inc., USA). Recommended instrumental parameters and respective standard curves for determination of individual mineral and trace elements was used. Direct drying method was employed for the determination of moisture content in the sample [17]. 10g of homogenized sample was dried minimum of 12h in an hot air oven at 110°C until constant weight of the sample was obtained. The difference between initial weight and constantweight after drying was taken to be moisture lost and hence the moisture content of sample and the results are expressed as g/100 g fresh weight of sample. Ash content of the sample was calculated after heating the sample at 500°C for 2 h in muffle furnace (SLV Scientific, India). All samples were analyzed in triplicate.

3. RESULTS AND DISCUSSION

3.1 UPLC analysis of phenolic compounds

The main objectives of this research were the separation, identification and quantification of the polyphenolic compound present in the wheat varieties. Simultaneous determination of various polyphenolic classes were possible by the use of UPLC with tunable UV detector. Table 1 and Fig. 1 depicts the complete chromatogram of separated certified REFERENCES standards (CRMs) of polyphenols at the wavelength of 280nm. By the use of a C18 column with 1.7µm particle size gave the opportunity to good resolution and faster separation with and high throughput analysis as compared to conventional HPLC columns where the more time required [18-19]. Also, there was low consumption of elution solvent due to usage of low flow rate (0.2 mL/min) in UPLC compared to conventional HPLC flow rate which is about 0.5-2.0mL/min (Fig.2). The very most important step in the analysis is sample preparation. It was very complex process due to existence of different structural complexity of various phenolic compounds, hence the process is made it difficult [20].

Table 1: Separation of 22 phenolic compounds using UPLC with their retention times (RT)

Sl. No.	Name of compounds	Retention time (RT)
1	Gallic acid	3.463
2	Resorcinol	4.301
3	3,4 Dihydroxybenzoic acid	5.443
4	3,4 Dihydroxybenzaldehyde	6.350
5	Vanillic acid	9.700
6	Catechin	10.063
7	Syringic acid	10.820
8	Caffeic acid	10.992
9	p-Coumarinic acid	15.112
10	Ferrullic acid	15.937
11	Sinapic acid	16.118
12	Protocatechuic acid	17.822
13	Rutin	19.515
14	Naringin	19.837

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15	Rosmarinic acid	21.760
16	Trans- cinnamic acid	22.457
17	Myrecitin	24.135
18	Luteolin	29.296
19	Quarcetin	29.603
20	Naringenin	30.264
21	Apigenin	32.517
22	Galangin	34.183

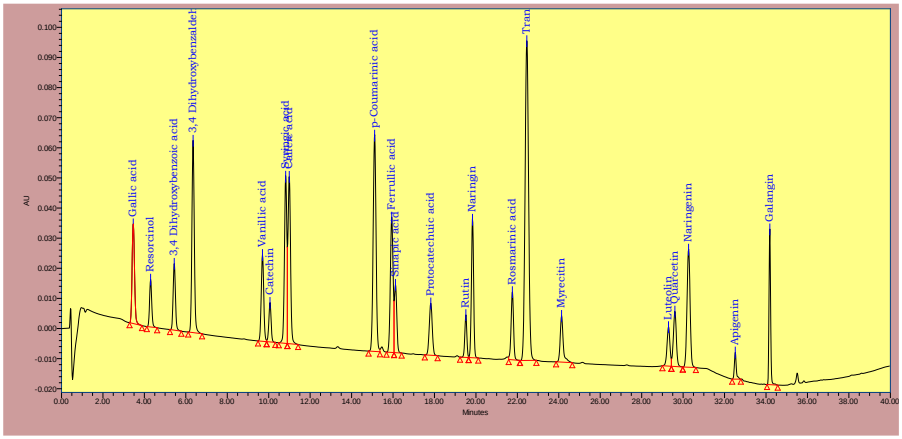

Figure 1: UPLC chromatogram of a certified REFERENCES standard mixture of 22 phenolic compounds at 280 nm.

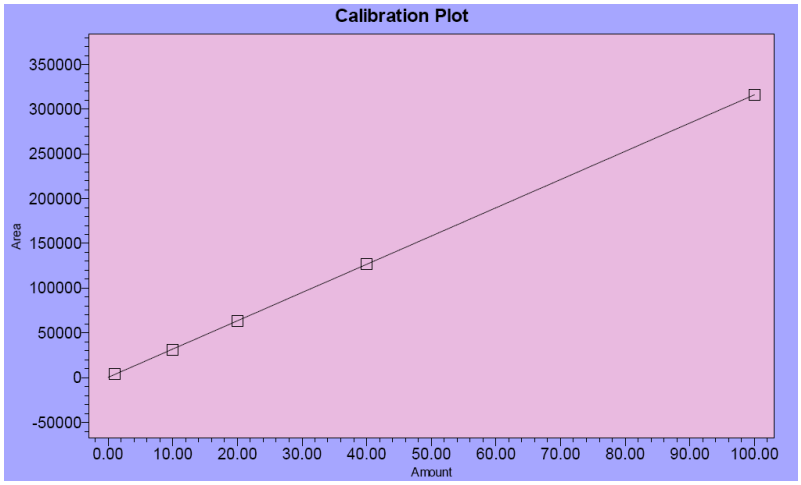

Figure 2: Typical UPLC calibration plot of certified REFERENCES standard (0-100ppb)

In the present study, a relatively much complicated protocol was used. Four phenolic acids namely ferullic acid, caffeic acid, syringic acid and p-coumarinic acid were identified and quantified by UPLC-TUV in four different wheat varieties. In the table 2, the distribution and composition and of the four detected phenolic acids were listed. Among wheat varieties, there were significant differences in the individual phenolic acid content was observed. The varieties namely, UAS-316 and DWR-162 had the highest quantities of ferulic acid at 210.00 and 142 mg/g dry weights, respectively. The remaining other two varieties i,e., DDK-1025 and GW-332 have showed the concentration of 112 and 65 mg/g dry weights respectively. Ferulic acid has been related with anti-diabetic, neuroprotective and anti-inflammatory properties, and acts as antioxidant that prevents cellular damage [21]. The UAS-316, GW-

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332, DWR-162 and DDK-1025 varieties had the highest levels of caffeic acid (126, 95, 64 and 64mg/g, respectively). There is notable interest in the content of caffeic acid in plants, particularly by the use of 3,4-dihydroxybenzoic acid (3,4-DHBA) and 4-HBA as a precursor for polyester as an potent antioxidant [22].

Table 2: Phenolic acid identified in wheat varieties using UPLC (mg/g dry matter)

Sl. No.	Name of elements	DWR - 162	UAS - 316	DDK - 1025	GW - 332
1	Ferullic acid	142	210	112	65
2	Caffeic acid	64	126	45	95
3	Syringic acid	16	29	35	60
4	p-Coumarinic acid	49	61	110	45

All the wheat varieties contained the considerable amount of syringic acid and had the highest concentration at 60 mg/g in GW-332 and lowest concentration of 16 mg/g in DWR-162. The other varieties viz., DDK-1025 &UAS-316 also contained notable amount of syringic acid i.e the concentration of 35 and 29 mg/g respectively. The three varieties DDK-1025, DWR-162 and GW-332 found to contained the p-coumarinic acid at the concentration of 110, 49 and 45 mg/g respectively , but it was absent in the UAS-316 variety. In general, ferullic acid and caffeic acids were the two most abundant phenolic acids present in varieties of wheat. However, the phenolic acid profiles of varieties of wheat i,e DWR-162, UAS-316, DDK-1025, and GW-332 differed.The phenolic acid namely, syringic acid was the less abundant in all the four varieties of wheat and was related with a disease preventive mechanism against various pathogenic microorganisms [23]. In order to understand the mechanism of action of phenolic acids in the human body exhaustive research efforts are required. Differences in polyphenolic acid composition among four wheat varieties might have been due to differences in climatic environmental change and soil properties [24-25].

3.2 Minerals and trace elements analysis

Ten minerals were determined in all the four wheat varieties (Table 3), including 5 major elements (Na, P, K, Ca and Mg) and 5 trace elements (Cu, Fe, Zn, Mn and S). The concentration of Na varied greatly between concentrations observed in DWR-162 (0.24%) and DDK -1025 (0.40%) varieties and GW-332 and UAS-316 showed 0.30 & 0.35 % respectively. All the four wheat varieties showed the concentrations of P in the range between 0.09 - 0.22%. The UAS-316 varietyhad showed P concentration 0.09% and the DWR-162 showed 0.22%. All the considered wheat varieties showed the concentration of K in the range between 0.20 - 0.34%. The lowest Ca concentrations (0.49%) were found in the UAS-316 and the GW-332 variety had the highest concentrations (1.00%). As regards to Mg, two varieties of wheat (GW-332 and UAS-316) had a lower concentration than DDK -1025%, which contrasted with the value 0.67% found in DWR-162 variety.

Table 3: Mineral, trace element concentrations and proximate composition for the wheat varieties analyzed.

Sl. No.	Name of elements	DWR - 162	UAS - 316	DDK - 1025	GW - 332
1	Sodium (Na, %)	0.24	0.35	0.40	0.30
2	Phosphorous (%)	0.22	0.09	0.19	0.10

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3	Potassium (K, %)	0.20	0.29	0.34	0.22
4	Calcium (Ca, %)	0.84	0.49	0.60	1.00
5	Magnesium (Mg, %)	0.67	0.23	0.48	0.12
6	Copper (Cu, ppm)	33.00	28.00	35.00	22.00
7	Iron (Fe, ppm)	731.00	750.00	6429.00	4298.00
8	Zinc (Zn, ppm)	339.00	290.00	338.00	265.00
9	Manganese (Mn, ppm)	137.00	142.00	152.00	168.00
10	Sulphur (S, %)	0.10	0.05	0.07	0.03
11	Moisture (%)	12.89	11.57	12.11	10.41
12	Total ash (gm)	0.027	0.050	0.109	0.065

Wheat varieties had the concentration of Fe between 731 and 6429 ppm. The DWR-162 showed lower range and DDK -1025 variety showed higher Fe concentrations, while the UAS-316 and GW-332 showed values of 750 and 4298ppm respectively. The concentrations of Cu contents in the varieties analyzed varied between 22 and 35ppm observed in the GW-332 and DDK-1025 varietiesrespectively. But other two varieties namely UAS-316 and DWR-162 presented concentration of Cu of 28 and 33ppm respectively. The Zn concentrations in the targeted wheat varieties were 265, 290, 338 and 339 ppm i,e., in the varieties GW-332, UAS-316, DDK -1025 and DWR-162 respectively. The concentrations of Mn were also vary among the varieties considered, varying between 137.00ppm for DWR-162, 142.00ppm for UAS-316, 152.00ppm for DDK -1025 and 168.00ppm for GW-332 varieties(highest Mn concentration). A notable variation in S concentrations in all the varieties was observed. The variety DWR-162 showed higher concentration (0.10%) and other three variety verities viz., GW-332, UAS-316 and DDK -1025 showed lower S levels i.e. 0.03, 0.05 and 0.07 % respectively. The contents of moisture in all the varieties ranged from 10.41 to 12.89 %. Similarly ash content was in the range of 0.027 to 0.109 gm.

In general, from a nutritional point of view, wheat is a good basket of nutrients especially in minerals and trace elements. The whole-wheat flour consumption (100 g) represents 33.5% of the RDI (recommended dietary intake) for P in persons older than 20 years and 36.3% and 27.7% of the RDI for Mg in females and males older than 33 years respectively [26]. The 100 g of wheat intake contributes to the K accounts for 9.3% of the AI (adequate intake) for adults to this element [27], while the Ca intake contributes to the 3.5% of the RDA for adults, and to the Na intake 0.7% of the AI for adults, which was low. The ratio of K/Na was very minimum (0.023) as compared to the values found in most of the other foods, which is very important aspect in the control and treatment of cardiovascular diseases and hypertension [28]. For the consumption of whole wheat higher levels of trace elements were found, which contributes for the Mn intake. So, whole-wheat flour consumption (100g) depicts 97.9% and 125.1% of the AI for Mn for males and females respectively [29]. The considerable amount of trace elements viz., Cu, Fe, S and Zn were also observed, which was in the range of 20% and 50% of the RDA recommended for the individuals of females and males [30]. Apart from this most of the defences from antioxidants depends on micronutrients.

It is proved that several antioxidant enzymes contained mineral and trace elements viz., catalase depends on Fe, glutathione peroxidase on Se, superoxide dismutase depends on Mn, Cu and Zn and magnesium is also present in significant amount in mitochondria which is have very important role in energy transfer [31]. The small differences were observed in the concentrations of mineral and trace element in the report of literature which might be due to genotype and effect of environment. Besides the results, it is very much crucial to consider the type of sample analyzed.Therefore, higher levels of mineral and trace elements were

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found in whole grains as compared to flour sample, which may be due to higher concentration of minerals accumulated in the outer kernel layers [32].

The results presented in this paper on Na were similar with most of the results published in the literature for T. turgidum or T. aestivum or [33]. The concentrations of P obtained in this paper which is lower than results reported by several scientists, who reported concentrations in the range between 0.40 and 0.50 % [34-35].Bibliographic reports on concentration of mineral K was agreed with the results presented in the table. The K concentrations were lower than the reports published by few researchers [36], they reported above the 0.50% concentrations. Several scientist observed that mean K concentrations equal, or a little lower, than the concentrations tabulated by us [37] for the whole-wheat flour.

Results obtained from our results for Ca was in the range between 0.49 and 1.00 % which obeys with several scientific investigations [38]. However, content of the Ca was slightly lower than other results presented in the literature [39-40].The data obtained for the concentration of Mg in this paper was very much identical with the most of the results published by various scientists [11,35,36,41]. But some of the published data from other authors [34, 37,40] were little higher than our reports. As per as concentrations of trace elements is concerned, results obtained for the concentration of Cu in our study were markedly indicated the lower than the most of the published data [11,33,34,35].

As compared to data found in the literature [34,35,37], the Fe results obtained by us were slightly similar or a little higher. But some authors documented higher Fe concentrations [42] and lower Fe concentrations [33] found in wheat samples. Regarding the Zn concentrations in wheat varieties [37,41] is concerned, the concentration ranged in a relatively narrow interval (14-190 ppm), the results documented in this paper were slightly higher than this range. The data presented for Mn concentration were slightly lower to or similar than those documented by other researchers [35,41], and very much lower than other results presented in the literature report [11,34]. Similarly for the concentration of S in wheat variety is concerned, the same trend was observed by several investigators [36,37].

4. CONCLUSION

In the present work, a very powerful UPLC-TUV analytical tool has been developed and used to separate a total of twenty two certified REFERENCES standards of phenolic compounds present in four varieties in the wheat varieties, four of them being detected and quantified in these samples of wheat varieties. Due to the combination of UPLC with TUV, it combines the advantages of C18 column (a small particle size) provided a higher resolution and made possible the separation of four phenolic acids with more sensitivity and accuracy. The developed chromatographic method for wheat phenolics are useful for the analysis and quantification of phenolic compounds in wheat varieties that those contain phenolic compounds in small quantities and could be utilized in the future for quantification purposes. Our results showed that an important differences exists among wheat varieties for minerals and trace elements composition. Furthermore, differences in the varieties significantly influenced their biochemical composition of minerals and trace elements. The considerable quantities of Na, P, K, Ca, Mg Cu, Fe, Zn, Mn and S was observed in wheat varieties.

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EVALUATION THE EFFECT OF ANTIOXIDANT AND ANTIDIABETIC PROPERTY OF HERBAL PLANT EXTRACT (*A. indicum)* ON NIDDM.

Lavanya L[1], Vadamalai Veeraraghavan[1]Renuka Srihari[2]
[1] School of Biochemistry, REVA University Bengaluru- 560064
[2]Department of Biochemistry, Maharani Lakshmi Ammanni College for Women, Bengaluru-560003, India.

ABSTRACT: *Abutilon indicum* a traditional Indian phytomedicine plant used for the treatment of several diseases such as Diabetes mellitus, Cardiac diseases and anti-inflammatory. The aim of the study is to evaluate whether *A. indicum* improves insulin sensitivity and determine its active role in bioactive secondary metabolites. In a normal tissue, Aldose reductase an enzyme, has low substrate affinity for glucose, so the conversion of glucose to sorbitol is less catalyzed. Hence Polyol pathway is used to catalyze the reduction of glucose to sorbitol. In Diabetes mellitus, the increased availability of glucose in insulin-insensitive tissues such as lens, nerve and retina lead to increased formation of sorbitol through polyol pathway. Hydrolysis of starch by pancreatic α-amylase and uptake of glucose by intestinal α-glucosidasecauses sudden rise in blood glucose levels or hyperglycemia in type 2 Diabetes mellitus [T2DM] patients. Various studies on plant extract have been performed to confirm the antioxidant assay and antidiabetic assay. A review study on the plant is provided to understand its medicinal properties.

Keywords*: Abutilon indicum,* Aldose reductase, antidiabetic activity, antioxidant assay

***Correspondence author E-mail** :l.lavanya11@yahoo.com, @Lavanya **Tel :** 8197673047
Veeraraghavan@reva.edu.in @ Dr V. Veeraraghavan **Tel:** 9880241577
rh_1thesis@rediff.com @ Dr Renuka Srihari **Tel:** 9740818539

1. INTRODUCTION

Diabetes mellitus is a complex disorder which involves disturbance of carbohydrates, fat and protein metabolism. It is caused due to the defect in insulin secretion. The metabolism of fats, protein and carbohydrates targeted on tissues is due to deficient action of insulin. The chronic hyperglycemia will lead to failure of different organs, damage and dysfunction especially eyes, kidneys, nerves, blood vessels and heart [1]. Symptoms of Hyperglycemia are polydipsia, polyuria, weight loss, polyphagia, and blurred vision. Hypertension and abnormalities of lipoprotein metabolism are most commonly found in diabetic patients [1]. There are two main types of Diabetes namely; Type 1 (Insulin-dependent) and Type 2 (Non-insulin-dependent) diabetes.

Type I Diabetes mellitus is an autoimmune disorder caused due to the destruction of β cells in pancreas [1]. It is also known as Insulin dependent Diabetes mellitus or early onset diabetes or juvenile diabetes as it is often found in teenagers [2]. The development of type 1 diabetes is also influenced by genetic, immune and environmental factors. Theactivation of abnormal mediated T-cells in immune response causes Insulitis, which involves inflammation of the β cells in pancreas with symptoms such as anxiety, hunger, nausea, sweating, palpitations, trembling, blurred vision, weakness etc. Type I Diabetes can be managed by daily injections of insulin, diet planning and screening for complications like Nephropathy, neuropathy, Retinopathy, Hyperlipidemia, Hypertension [3].

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Type II Diabetes mellitus is non- insulin independent diabetes mellitus/NIDDM/Maturity onset/T2DM/Type II predominantly occurring in the older people often has a greater number of α-cells with lower number of β-cells increased gluconeogenesis in the liver, express high insulin sensitivity and reduce uptake of glucose in adipose tissue and muscles in insulined conditions. Non-insulin dependent Diabetes mellitus (NIDDM)/Type 2 diabetes is also referred to as maturity onset diabetes because it is commonly found in people over the age of 40. Type 2 Diabetes mellitus is characterized by impaired secretion of insulin, hyperglycemia and glucose intolerance. It is a low-grade inflammatory disease caused by long term immune system imbalance or excess of nutrients associated with fat and obesity. Hypoglycemia is also caused due to excess insulin secretion by insulinomas cell [4]. Commonly, complications occur in arteries, eyes and kidneys. Inflammation is majorly considered for association of T2DM as T-lymphocytes plays an important role in pathogenesis of T2DM [5,6]. Diabetes mellitus chronic complication includes mainly accelerated development of cardiovascular disease, limb amputation, loss of vision etc.

All of these complications contribute to excess morbidity and mortality in individuals. That is, the burden of Diabetes mellitus and its complications will affect many more individuals than currently anticipated and cost to our society. Hypoxia refers to pathological condition where oxygen supply is limited in tissues hence can be recognized that hypoxia is involved in T2DM. It also plays a critical physiological role as transcriptional activator. The high rate of β cells are due to apoptosis which affects the factors such as blurred vision, nephropathy, neuropathy, foot ulcer. [7].

Diagnosis of diabetes can be done through blood test such as glycated hemoglobin or oral glucose intolerance test. Clinical management includes like dietary change, medication, regular exercise and augmentation of insulin [8] In diabetic patients, the profound vascular disorder is due to hyaluronidase and hyaluronan plasma level which were found to be high with reduced glycocalyx volume. Hyperglycemia can also degrade enzymes and activates glycocalyx [9,10]. This can be prevented through reducing cholesterol level, regular exercise, healthy diet etc. [11].

Abutilon indicum commonly called as ‘Atibalaa’, a small perennial shrub which belongs to malvaceae family. *A. Indicum* is usually found in tropic and subtropical regions. Various parts of the plant have many medicinal properties hence widely used in the form of natural product to cure various diseases. Few physical characteristics of the plant can be mentioned with height reaching upto 3m, stout stem, yellow color flowers which blossom in the evening, velvety leaves, stellate -hairy dark brown seeds ranging 3-5mm in size [12]. Properties of *A. Indicum* is used in medicines such as Aphrodisiac, diuretic, Demulcent, astringent, expectorant, tonic, anti-inflammatory, anthelmintic, laxative, antioxidant, analgesic and to treat leprosy, ulcers, headaches, anti-diabetic, anti-glycemic, anti-lipidemic, gonorrhea, bladder infection and anticholestatic. [13]

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Fig 1: Morphology of *Abutilon indicum*

Kingdom	Plantae
Subkingdom	Tracheobionta
Order	Malvales
Family	Malvaceae
Genus	Abutilon
Species	*Indicum*

Table:1 Scientific classification of *Abutilon indicum*

2. MATERIALS AND METHODS

2.1 Plant material

The plant sample *A. indicum* was collected from "Krishnendra Botanical plant nursery" Lalbagh – Bengaluru. The collected plant sample was dried by direct sunlight drying method.

2.1.1 Extraction

-Weighed 20 g dried powder of powder was dissolved in 100 ml of solvent in 500 ml beaker and was covered with Aluminum foil.

-Then the beaker was kept on hot water bath at 50º C for 4 hours.

-After incubation period the extract was filtered with whatmann filter paper and the filtrate was collected in 50 ml beaker. Residue present over the filter paper was discarded and filtrate was taken for further use.

-Then the filtrate was kept at 50 ºC for few hours until the extract gets completely dried and turn into semisolid form.

-10mg of semi-solid crude extract was taken from the total yield and dissolved in 1ml of methanol in an Eppendorf tube to make it 10mg/ml stock solution.

-After that working concentration of the test sample i.e. 0, 10, 20, 40, 80, 160 and 320µg/ml were prepared from the stock solution.

2.2 Phytochemical analysis:

The preliminaryPhytochemical screenings of plant extract of *A. indicum*was carried out accordingly to the standard methods. The presence of important phytochemicals was evaluated by using standard procedures [14] with 10mg/ml concentration of plant extracts.

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2.2.1 Test for Alkaloids: 0.2ml of sample was taken and 0.2ml of HCl was added. To this 2-3 drops of Dragendoff's reagent was added and the appearance of orange or red precipitate and turbid solution indicates the presence of alkaloids.

2.2.2 Test for Carbohydrates: 0.2ml of sample was mixed with few drops of Molisch's reagent (α-napthol dissolved in alcohol). 0.2ml of Sulphuric acid was added along the sides of the test tube and observed for the appearance of a purple color ring for positive test.

2.2.3 Test for Flavonoids: 0.2ml of plant extract was taken in a test tube and mixed with dilute sodium hydroxide solution. To this diluted hydrochloric acid was added. Observation of yellow solution that turn colorless later would indicate the presence of flavonoids.

2.2.4 Test for Glycosides: 0.2 ml of sample was mixed with 0.2ml of chloroform. 0.2ml of acetic acid was added to this solution and the mixture was cooled on ice. Sulphuric acid was added carefully and the color change from violet to blue to green indicates the presence of steroidal nucleus (A glycone portion of glycoside).

2.2.5 Test for phenols: To 1-2ml of extract ,0.5 ml of $Fecl_3$ is added. Color changes to green this indicates the presence of phenols.

2.2.6 Test for Saponins: To 0.5g of extract 5ml of distilled water was added in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

2.2.7 Test for Steroids: 0.2ml of sample was mixed with 0.2ml of chloroform. To this 0.2ml of concentrated Sulphuric acid was added. The appearance of red color in the lower layer of chloroform indicates the presence of steroids.

2.2.8 Test for Tannins: 0.2ml of plant extract was mixed with 2ml water and heated on water bath for 10mins. The mixture was filtered and ferric chloride was added to the filtrate and observed for dark green solution which indicates the presence of tannin.

2.2.9 Test for Terpenoids: 0.2ml of plant extract was taken in a test tube with 0.2ml of chloroform. To this, concentrated Sulphuric acid was added carefully to form a layer. Presence of reddish-brown color at the interface would show the presence of terpenoids.

2.2.10 Antioxidant Activity

Method used for antioxidant activity was ABTS free radical scavenging assay, DPPH free radical scavenging assay.

2.2.11 ABTS free radical scavenging assay

Free radicals are generally very reactive molecules possessing an unpaired electron which are produced continuously in cells either as by-products of metabolism or by leakage from mitochondrial respiration [15]. The free radicals produced in-vivo include the active oxygen species such as super-oxide radical O_2^-, hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

During metabolism, oxygen consumption involves the constant generation of free radicals and reactive oxygen species (ROS). H_2O_2 and O_2 can interact in the presence of certain transition metal ions to yield a highly- reactive oxidizing species, the hydroxyl radical (OH) [16]. The hydroxyl radical, one of the ROS, is an extremely reactive free radical formed in biological systems and reacts rapidly with molecules found in living cells, for example, sugars, lipids, DNA bases, amino acids [17]. Oxygen free radicals have been shown to be responsible for many pathological conditions [18]. Free radicals and Reactive Oxygen Species (ROS) cause DNA damage, lipid per-oxidation, protein damage. They are known to be involved in the pathogenesis of a wide variety of clinical disorders as cancer, cardiovascular diseases, inflammatory diseases, asthma and aging [19,20]. Free radicals like

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the hydroxyl radical, hydrogen peroxide, superoxide anion mediate components of the inflammatory response, with production of migratory factors, cyclic nucleotides and eicosanoids. Superoxide radicals amplify the inflammation process, increasing vascular permeability, adhesion of polymorphonuclear leucocytes to the endothelium and stimulation of platelet aggregation [21].

Some enzymatic and non-enzymatic systems exist in the bodies which are involved in the detoxification process like superoxide dismutase, catalase [22]. Aerobic metabolism entails the production of reactive oxygen species, thus there is a continuous requirement of antioxidants for their inactivation. Thus, the steady state of pro-oxidants and antioxidants may be disrupted in favor of the former, leading to oxidative stress, which may affect all types of biological molecules [21].

Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves.

Plants possess antioxidant principles. Various classes of phytochemicals have been shown to have antioxidant property which is due to the presence of substituted groups such as carbonyl, phenolic, phytyl side chain, electron withdrawing group, electron donating group etc. Phenolic antioxidants donate hydrogen to the radical and convert it to stable non-radical product [23] ABTS assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical, a process which is associated with a change in absorption which can be followed spectroscopically. The relatively stable ABTS radical has a green color and is quantified spectrophotometrically at 734nm.

$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ Sample)}{OD\ of\ Control} \times 100$$

2.2.12 DPPH ASSAY

The generation of reactive oxygen species (ROS) beyond the antioxidant capacity of the biological system give arise to oxidative stress. Free radical oxidative stress has been implicated in the pathogenesis of a variety of human diseases such as diabetes mellitus, hypertension, atherosclerosis, inflammation, cancer etc. [24]. A group of substance are antioxidant when it is present at low concentration significantly they delay or inhibit by oxidative processes, while they oxidized by themselves. The effect of antioxidant in biological systems will exerts different mechanisms including metal ion chelat ion there by eliminating potential free radicals, sparing of antioxidants as co-antioxidants ,electron donation as reducing agents [25]. Antioxidants lower the burden of free radicals and they have the ability to take up the free radicals and reduce the free radical and make it stable.

The DPPH scavenging assay is a simple chemical experiment for the primary evaluation of any compound for its simplicity and low cost for free radical scavenging activity [26, 27].

DPPH [1,1-diphenyl-2-picryl hydrazyl] is a stable free radical with purple colour. Antioxidants reduces DPPH to 1,1-diphenyl-2-picryl hydrazine, colourless compound which is measured at an absorbance of 590nm.

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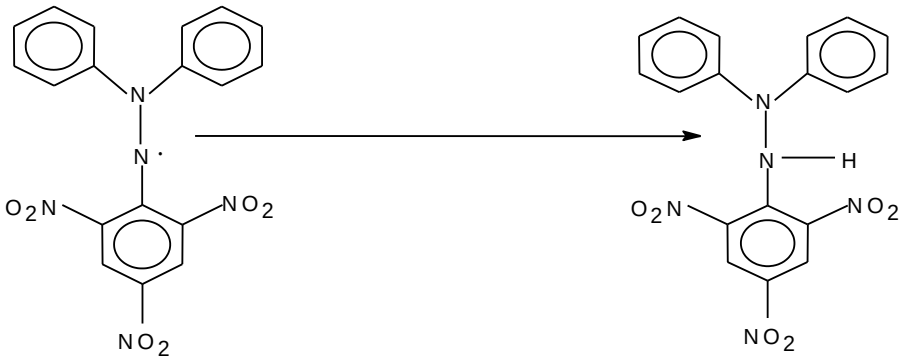

1,1-diphenyl-2-picryl hydrazyl **(Purple colored)**

1,1-diphenyl-2-picryl hydrazine **(Colorless)**

$$\%\ Inhibition = \frac{(OD\ of\ Control - OD\ of\ Sample)}{OD\ of\ Control} \times 100$$

2.2.13 ALDOSE REDUCTASE INHIBITION ASSAY

Diabetes mellitus is an endocrine disorder in which glucose metabolism is impaired because of total loss of insulin after destruction of pancreatic β-cells in insulin dependent diabetes mellitus and inadequate release of insulin from the pancreatic β-cells or insensitivity of target tissues to insulin in non-insulin dependent diabetes mellitus. Of the currently available therapies for NIDDM, only the sulphonyl urea are used to stimulate the β-cells to secrete more insulin. Sulphonyl urea act at a proximal stage in the β-cells stimulus secretion coupling cascade: they close plasma membrane ATP-sensitive K^+ and the consequent decrease in the K^+ efflux depolarizes the cells, leading to Ca^+ influx via voltage-operated **Ca^+channels [28].**

One of the major physiological effects of insulin is to promote the uptake, metabolism and storage of glucose in adipose tissue and skeletal muscle. Insulin stimulates phosphorylation of insulin receptor substrates [IRS-1/2] by the insulin receptor kinase, which leads to activation of PI3 kinase and downstream kinases such as PKB and protein kinase C isoforms [29]. Activated PKB has been shown to induce the translocation of Glut 4 to the cell surface and stimulate glucose transport in muscle and fat cells, whereas it phosphorylates and inhibits GSK-3. Inhibitor of GSK-3 enhances insulin signaling to glucose transport in L6 muscle cells and 3T3L1 adipose tissue cells. Increases in intracellular Ca+ are sufficient to stimulate insulin release from β-cells secretary granules.

Aldose reductase has a major role in the conversion of glucose to sorbitol in the polyol pathway as it plays a key role to catalyze to reduce glucose to sorbitol, it has got low affinity substrate for glucose and gets less catalyzed [30,31]. However, in Diabetes mellitus insulin gets increased amount of glucose-insensitive tissues like retina, lens and nerves through polyol pathway which leads to the formation of sorbitol.

2.2.14 α-Amylase Inhibition Assay

Proteinaceous inhibitors of the important digestive enzyme α-amylase [1,4,α-D-glucanhydrolyases, EC 3.2.1.1] are wide spread in plants, notably in cereals (wheat, barley) and legume (beans) [32]. The α-amylase catalyzes the hydrolytic degradation of polymeric carbohydrates such as amylose, amylopectin and glycogen by cleaving 1, 4, α-glycosidic bonds. In polysaccharides and oligosaccharides, several glycosidic bonds are hydrolyzed simultaneously; maltotriose is converted to maltose and glucose. [33]

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α-amylases are of 2 types the and salivary α-amylase and pancreatic α-amylase. In humans, the digestion of starch involves several stages. Initially, partían digestion by the salivary amylase results in the degradation of polymeric substrate into shorter oligomers. Further hydrolysis takes places in guts by converting pancreatic α-amylase into maltose, maltotriose enzyme and small malto-oligosaccharides [34,35]. Breaking down of glucose is due to the hydrolyzing dietary starch to maltose in the absorption. α-amylase inhibited to get reduced form of unfavorable high postprandial blood glucose peak in diabetics. [36,37]

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis:

Preliminary biochemical screening of methanolic extract of *A.indicum* suggested that various phytoconstituents which has medicinal properties .As shown in the table 2

Table 2: Preliminary test for phtochemical

Tests	Result
1) Alkaloid Test (Dragendoff's Test)	+
2) Carbohydrate Test (Molisch's Test)	-
3) Tannin Test	+
4) Terpenoids Test	+
5) Steroid Test	-
6) Saponin Test	-
7) Flavonoid Test	-
8) Phenols Test	+
9) Glycosides Test	+
10) Cardiac Glycosides Test	+

3.2 ABTS free radical scavenging assay

In ABTS assay, sample showed dose-dependent ABTS radical scavenging activity with IC50 value of 70.78µg/ml when compared to standard Quercetin with IC50 value of 1.227µg/ml shows in the Table 3.

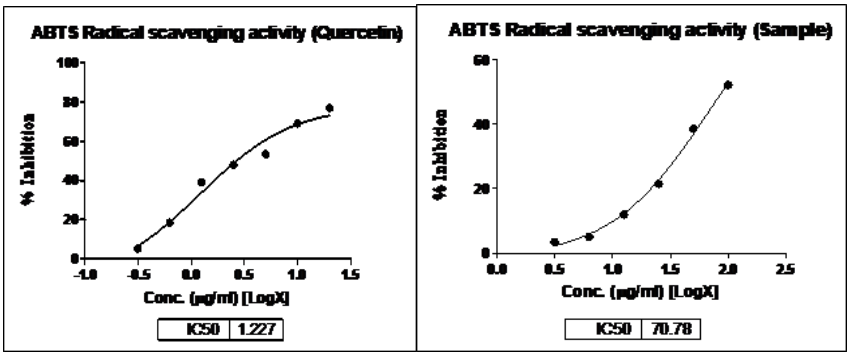

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Fig. 2: Graphical representation ABTS activity of Quercetin and *A.indicum*

Table 3: Concentration of Quercetin and *A.indicum*

Control	Conc. (µg/ml)	Absorbance (734nm)	% Inhibition	IC_{50} (µg/ml)
Control	0	0.860	0	0
Quercetin	0.3125	0.815	5.30	1.227
	0.625	0.701	18.48	
	1.25	0.524	39.10	
	2.5	0.475	44.80	
	5	0.400	53.46	
	10	0.266	69.07	
	20	0.199	76.92	
Sample	3.125	0.831	3.42	70.78
	6.25	0.817	5.07	
	12.5	0.757	11.99	
	25	0.675	21.51	
	50	0.528	38.67	
	100	0.411	52.27	

3.3 DPPH Assay

In DPPH assay, sample showed dose-dependent DPPH radical scavenging activity with IC50 value of 99.12µg/ml when compared to standard Quercetin with IC50 value of 1.7µg/ml showed in the Table 4

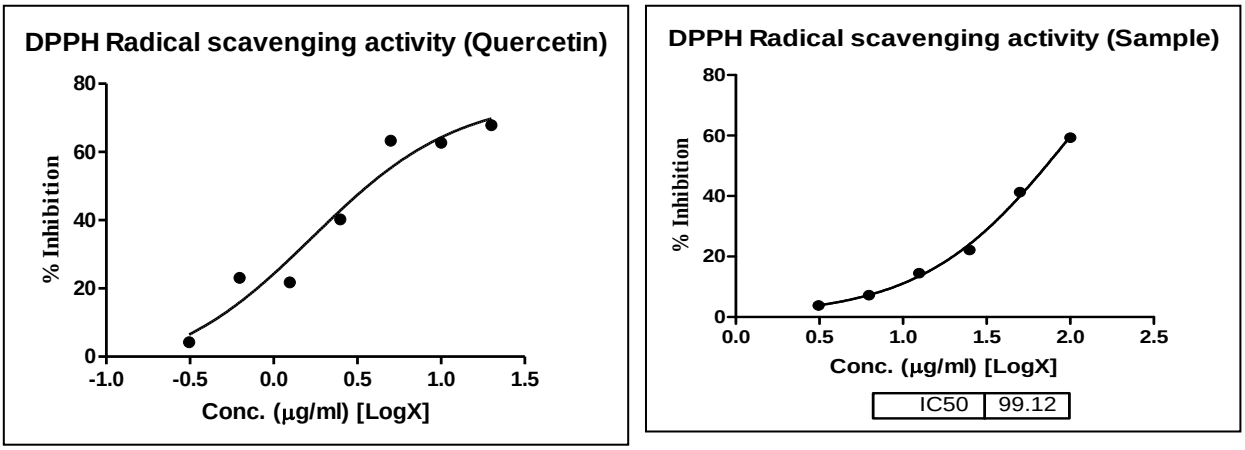

Fig.3: Graphical representation of DPPH scavenging avtivityof Quercetin and *A.indicum* sample

Table 4: **Concentration of Quercetin and *A.indicum***

Sample Name	Conc. (µg/ml)	Absorbance (590nm)	% Inhibition	IC_{50} (µg/ml)

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Control	0	0.252	0	0
Quercetin	0.3125	0.241	4.2063492	1.70
	0.625	0.194	23.095238	
	1.25	0.197	21.785714	
	2.5	0.151	40.238095	
	5	0.093	63.293651	
	10	0.094	62.698413	
	20.000	0.081	67.857143	
Sample	3.125	0.242	3.8492063	99.12
	6.25	0.234	7.2222222	
	12.5	0.216	14.484127	
	25	0.196	22.142857	
	50	0.148	41.269841	
	100	0.103	59.325397	

3.3 Aldose Reductase Inhibition Assay

The standard copper sulphate showed significant inhibitory activity with IC50 values of 13.60 µg/ml. similarly tested sample showed significant inhibitory activity with IC50 values of 135.8 µg/ml.

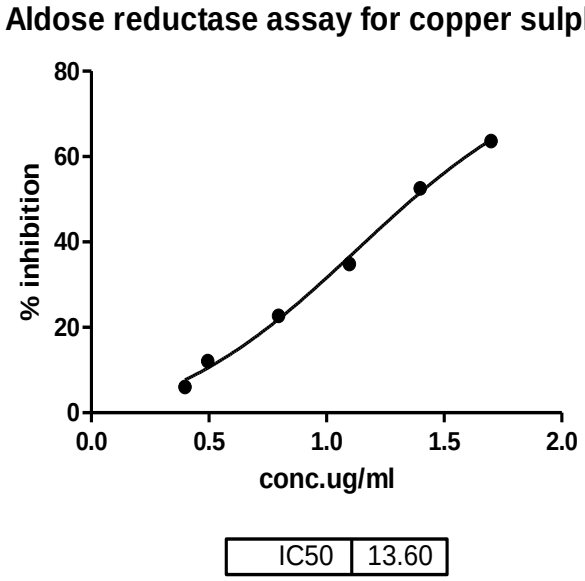

Fig. 4: Graphical representation on %inhibition on *A.indicum* (sample)

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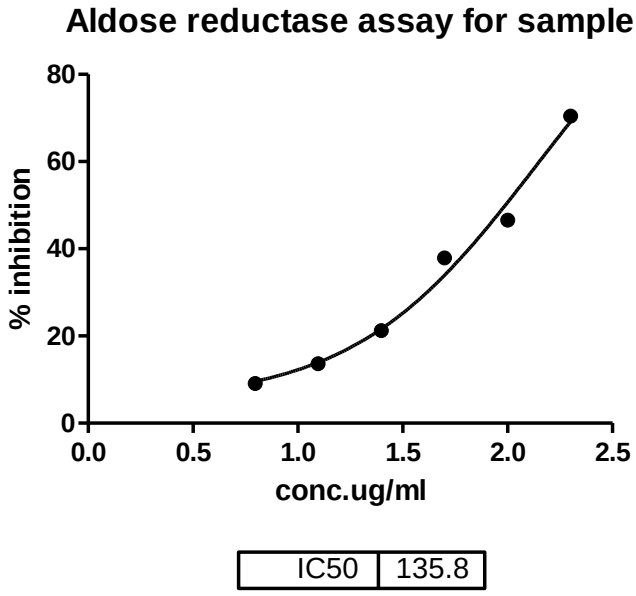

Fig. 5: Graphical representation on %inhibition on *A.indicum* (sample)

Table 5: Aldolase reductase activity of standard and test samples

Compound name	**Conc. µg/ml**	**OD at 340 nm**	**% Inhibition**	**IC_{50} µg/ml**
Control	0	0.495	0.00	
Copper Sulphate	2.5	0.465	6.06	13.60
	3.125	0.435	12.12	
	6.25	0.383	22.73	
	12.5	0.323	34.85	
	25	0.234	52.58	
	50	0.180	63.64	
Sample	*6.25*	0.450	9.09	135.8
	12.5	0.428	13.64	
	25	0.390	21.21	
	50	0.308	37.88	
	100	0.265	46.55	
	200	0.146	70.46	

3.4 α-Amylase Inhibition Assay

The Sample shows significant inhibition of Alpha-amylase with IC50 value of 92.50 µg/ml. The standard Acarbose showed inhibition of α—amylase with IC50 value of 2.454 µg/ml. Overall, these result suggests that the sample has very good antidiabetic activity. The IC50 values are listed in the Table 6.

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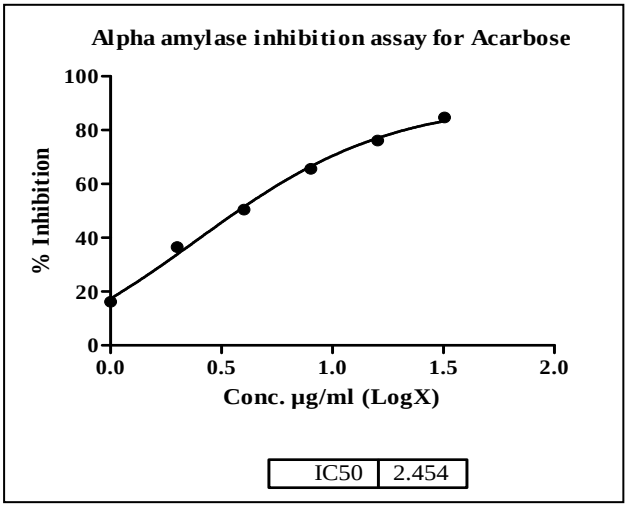

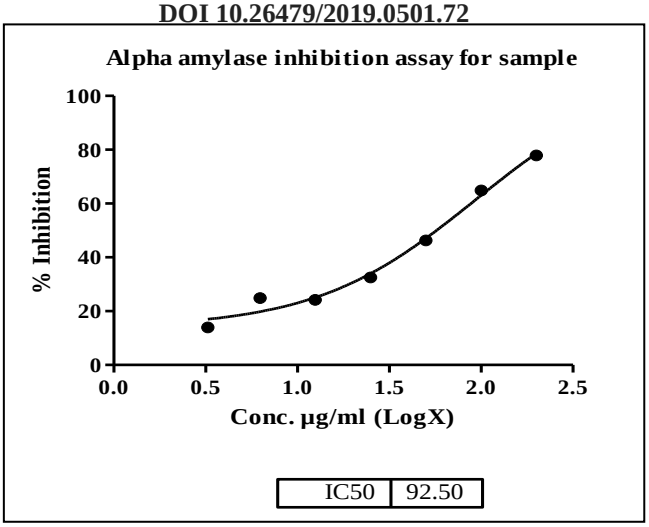

Fig. 6: Graphical presentation of α-amylase activity against acarbose and *A.indicum*

Table 6: Concentration on Sample

Sample Name	Conc. (µg/ml)	Absorbance (405nm)	% Inhibition	IC_{50} (µg/ml)
Control	0	0.97	0.00	0
Acarbose	1	0.81	16.19	2.45
	2	0.61	36.57	
	4	0.48	50.46	
	8	0.33	65.63	
	16	0.23	76.16	
	32	0.15	84.72	
Sample	3.25	0.83	13.92	92.5
	6.25	0.73	24.86	
	12.5	0.73	24.18	
	25	0.65	32.54	
	50	0.52	46.33	
	100	0.34	64.90	
	200	0.21	77.85	

4. CONCLUSION

Now a days, Diabetes is a major cause of concerns due to a large fraction of the population being affected. Even though effective treatment for the disease, there are lots of side effect associated with them. Hence, finding out a treatment is very important. Herbal medicine can be a good alternative for treatment. Hence, there is a need to test the glucose uptake on other species to find out a herbal alternative which has lesser side effect compared to the drugs available in allopathic medicine. Many herbs contain strong components to control diabetes. In the review, natural products classified into phytochemical analysis has some other categories have shown that antioxidant and antidiabetic potential through the plant extract.

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BIOCHEMICAL TEST DISCLOSE THE BACTERIAL DIVERSITY IN LAKE WATER

Charanraj TP[1*], Poonam Kumari[1], Subham Saha[1]
[1]Department of Biochemistry, School of Applied Sciences, REVA University, Bangalore, Karnataka-64

ABSTRACT: Lake water has many diverse forms of bacteria. The results of Gram staining, Oxidase, Catalase tests were discussed. With the help of gram staining test we conclude that all organisms are gram-positive because they all have retained the crystal violet color as they are having thick peptidoglycan layer, and with the help of the oxidasetest, we conclude that all bacteria of the sample produces cytochrome c oxidase enzyme which is primarily known for its function in the mitochondria as a key participant in the life-supporting function of ATP synthesis. Finally, with the help of Catalase test, we concluded that except one sample all gave the positive results.Catalase is constantly in battle against the effect of free radicals on the body. It transforms harmful superoxide radicals into hydrogen peroxide which later breaks down into water and oxygen.

KEYWORDS: Bacteria, Gram staining, Oxidase, Catalase, Coagulase, Indole, Cytochrome c oxidase.

***Corresponding Author**: Charan Raj T.P
Department of Biochemistry, School of Applied Sciences, REVA University, Bangalore, Karnataka-64.
Email Address:Charanraj.tp@reva.edu.in

1. INTRODUCTION

Microbial diversity is an important constituent of an overall global biological diversity present on this planet. Microbial diversity that we observe today is the consequence of nearly 4 billion years of evolutionary change. Apart from several niches present in earth, the freshwater lakes are important habitats for diverse microbial communities [1].Bacteria have distinct cell body shapes, ranging from spheres (cocci) to rods (bacilli) of various curvatures and helicities and to more exotic shapes, such as stars, formed by elaboration of prosthecae through polar growth. Bacteria can also produce a variety of appendages, such as pili or flagella, which show diversity in overall shape, length, and width as well as placement with respect to the cell body. Finally, bacteria can change morphology during their life cycle or in response to environmental conditions [2]. Microorganisms are present everywhere on Earth that will support life. These include habitats like soil, water, animal, and plants as well as virtually any structures made by humans [3]. Efforts are made to find out the bacterial diversity. There are different types of test to determine the bacterial species. These tests have various significance for example with the help of Gram Staining test,The Gram stain easily divides bacteria into two groups, Gram-positive and Gram-negative, on the basis of their cell wall and cell membrane permeability. The mechanism further implies that solvent decolorization causes significant damage to the cell surfaces of Gram-negative bacteria and only limited damage to Gram-positive bacteria. This suggests Gram-negative bacteria are more "leaky," causing these thin-walled lipid-rich cells to lose their crystal violet (CV) stain and appear red from the counterstain. Gram-positive cells, thick-walled and lipid-poor, appear blue from retaining the original CV [4-5]. Catalase test, the importance of the catalase reactions, by which hydrogen peroxide is broken down to water and oxygen, is well known

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for its application to the differentiation of gram-positive organisms [6].The oxidase reaction, based upon the ability of certain bacteria to produce indophenol by the oxidation of dimethyl-p-phenylenediamine and a-naphthol, was introduced by Gordon & McLeod (1928) to aid identification of gonococci, although specificity for this organism was not claimed [7]. Starch hydrolysis test, α-Amylases (E.C.3.2.1.1) are the enzymes that catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose,and maltotriose units [8-11]. Indole, a bacterial product of tryptophan degradation, has a variety of important applications in the pharmaceutical industry and is a biomarker in biological and clinical specimens. Yet, specific assays to quantitate indole are complex and require expensive equipment and a high level of training. Thus, indole in biological samples is often estimated using the simple and rapid Kovács assay, which nonspecifically detects a variety of commonly occurring indole analogs [12]. The methyl red (MR) test devised by Clark & Lubs (1915) has been used mainly to distinguish between coliform bacteria.The organisms are grown in a medium containing glucose, phosphate,and peptone, and after incubation at 30' for 5 days cultures are tested by the addition of a few drops of methyl red solution [13]. Voges Proskauer test, the ability to produce acetylmethylcarbinol (acetoin) from glucose, as demonstrated by the Voges-Proskauer (VP) test, is a valuable taxonomic characteristic, especially useful for differentiating members of the Enterobacteriaceae [14-17].Citrate is a ubiquitous natural compound which can be utilized as a carbon and energy source by many bacterial species. Usually involves the key enzyme citrate lyase, which catalyzes the cleavage of citrate into acetate (the end product) and oxaloacetate [18-21]. Urease test, Urease is an enzyme that hydrolyzes urea (carbamide) into ammonia and carbon dioxide and is produced by several bacterial species. Detection of urease activity has become an important tool for the diagnosis of *Helicobacter pylori* infections in association with chronic gastritis, which increases the risk of developing peptic ulcer [22-23].Coagulase test, the ability to clot plasma is generally accepted as the most reliable criterion for the identification of staphylococci [24]. The tube test, which demonstrates the production of free coagulase [25], is considered the definitive test for coagulase production [26]. The CAMP (Christic, Atkins, and Munch-Peterson) test has also been used as a simple and inexpensive test for diagnosis in which approximately 98% of the strains produce CAMP factor protein (Darling 1957, Jewes 1986, Philips 1980). Wilkinson and co-workers (1977) and Diperso and co-workers (1985) described the interactions between the CAMP factor and Staphylococcal Beta-hemolysin [27].

2. MATERIALS AND METHODS

In the First step,we collected the samples from the desired places like Yelahanka Lake and Kattigenahalli Lake. In the next step, we have made serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) so as to get proper colonies of bacteria.Right after this, we prepared the agar media (90ml) to culture the bacteria. Agar media preparation: - Peptone-0.5g, Agar-2.0g, Beef extract-0.3g, Yeast extract-0.3g, NaCl-0.5g. Sterilization: - Is a process that eliminates or removes all forms of life and biological agent present in the specified region. To name a few fungi, bacteria, protozoa, virus. Here the sterilization technique used is Wet Heat (autoclave). It's a device which uses high pressurize saturated steam at 121°C (249 Fahrenheit) depending on the size of the load and the contents. We carried out plating followed by incubation for 48 hours for the growth of culture.Later on, we streak the new agar plates from the stock culture. Subsequently, we obtained pure cultures. So we have performed the tests like gram staining, catalase, and oxidase testandresults were found to be positive.

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Gram stain test:- Cells are stained with crystal violet dye. Next, a Gram's iodine solution (iodine and potassium iodide) is added to form a complex between the crystal violet and iodine. This complex is a larger molecule than the original crystal violet stain and iodine and is insoluble in water. The decolorizer such as ethyl alcohol or acetone is added to the sample which dehydrates the peptidoglycan layer by shrinking and tightening it. The large crystal violet-iodine complex is unable to penetrate this tightened peptidoglycan layer and thus trapped in the cell of gram-positive bacteria.Conversely, the outer membrane of Gram-negative bacteria is degraded and the thinner peptidoglycan layer of Gram-negative cells was unable to retain the crystal violet-iodine complex and the color was lost. A counterstain, such as the weakly water soluble safranin, is added to the sample, staining it red. Since the safranin is lighter than crystal violet, it does not disrupt the purple coloration in Gram-positive cells. However, the decolorized Gram-negative cells are stained red.

Catalase Test: - The presence of catalase in a microbial or tissue sample can be demonstrated by adding hydrogen peroxide and observing the reaction. The production of oxygen can be seen by the formation of bubbles. This easy test, which can be seen with the naked eye, without the aid of instruments, is possible because catalase has a very high specific activity, which produces a detectable response, as well as the fact that one of the products is a gas.

Oxidase Test: - The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. We have used TMPD reagent. All bacteria that are oxidase positive are aerobic and can use oxygen as a terminal electron acceptor in respiration. This does not mean that they are strict aerobes. Bacteria that are oxidase-negative may be anaerobic, aerobic, or facultative; the oxidase negative result just means that these organisms do not have the cytochrome c oxidase that oxidizes the test reagent. They may respire using other oxidases in electron transport. Tests like starch hydrolysis, Indole, Methyl red and Voges Proskauer, Citrate Utilisation, Urease, Coagulase, and CAMP are performed later and published elsewhere.

3. RESULT AND DISCUSSION

3.1 Stock Culture

Figure 1-Stock culture from Yelahanka lake

Figure 2- Stock culture from Kattigenahalli

3.2 Streaked Plates

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Figure 3: Streaked plates from stock cultures cultures of of Kattigenahalli

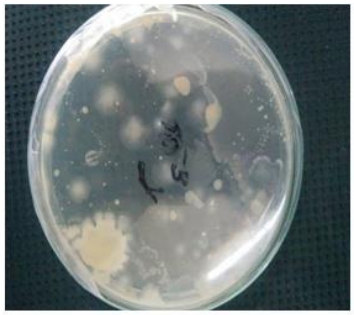

Figure 4: Streaked plates from stock Yelahanka Lake showing growth of bacterial colonies

3.3 Gram stain test:

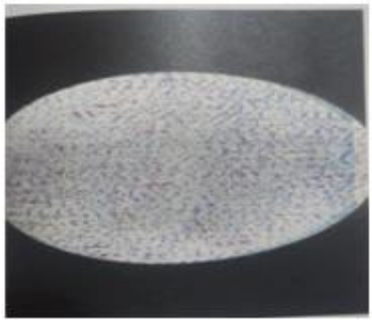

Figure 5. 10^{-1} dilution of the sample from Kattigenahalli lake indicates gram positive bacteria.

3.4 Catalase test:

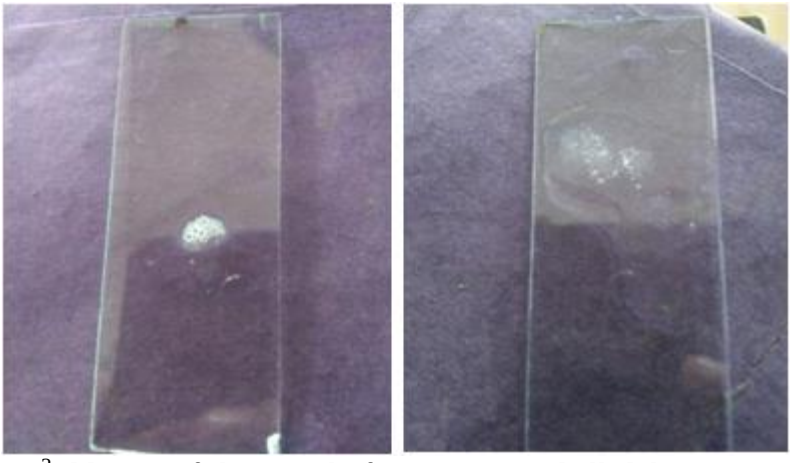

Figure 6. 10^{-3} dilution of the sample from Kattigenahalli lake Figure 7- 10^{-2} dilution of the sample from showing catalase enzyme(degradation of hydrogen peroxide)Yelahanka lake showing absence of catalase enzyme in bacterial colonies,

3.5 Oxidase test:

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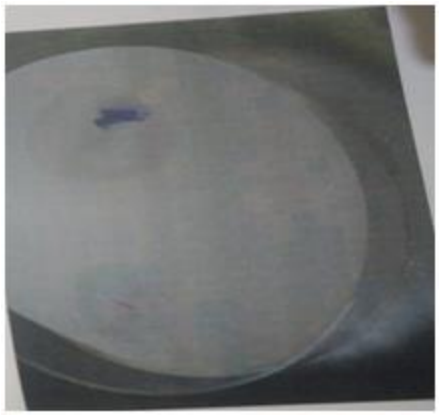

Figure 8. 10^{-3} dilution of sample from Yelahanka Lake indicating presence of Cytochrome c oxidase enzyme.

4. CONCLUSION

The water samples were collected from Yelahanka and Kattigenahalli lake to check the bacterial diversity. Gram stain test was performed in our laboratory to check the bacteria's whether Gram-positive or Gram-negative colonies. Catalase test when performed gave positive result except for one dilution (10^{-2}). Oxidase test results showed that the colonies were producing Cytochrome c oxidase enzyme. Other tests such as starch hydrolysis, Indole, Methyl red and Voges Proskauer, Citrate Utilization, Urease, Coagulase, and CAMP are to be performed in REVA University and published elsewhere. In the future, enzymes can be extracted from them, can be used in the production of traditional foods such as yogurt, cheese, and vinegar; biotechnology and genetic engineering, producing substances such as drugs and vitamins; agriculture; fiber retting; human and animal digestion; and biological control of pests.

CONFLICT OF INTEREST

None

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EXTRACTION AND ESTIMATION OF SECONDARY METABOLITES IN THE LEAF OF *OCIMUM SANCTUM*

Ramesh Kumar Kushwaha[1*], Sridhar Gowda K L[1], Shashi Kumar B S[1], Vinay Kumar D N[1]
[1]Department of Biochemistry, REVA University, Bangalore-64

ABSTRACT: *Ocimum sanctum* has so many medicinal properties like anti-oxidant, antimicrobial, antidiabetic, antifungal, anti-fertility, analgesic actions and they have been recommended for the treatment of bronchitis, skin disease, diarrhea, malaria and so on. It is cultivated for religious and traditional medicine purpose. *O. sanctum* leaves contain numerous phenolic compounds such as cirsilineol, circimaritin, isothymusin, apigenin and rosaneric acid. Here we were looking for extraction of total phenolic compounds and their anti-oxidant property. Leaves of *O. sanctum* were considered for the phenolic compound extraction in ethanol by using soxhlet method for extraction. Apart from phenolic compounds other secondary metabolites quantitatively analyse the presence of terpenoids , flavonoids and tannins. The quantitative analysis of phenolic constituents was determined using high-performance liquid chromatography and gallic acid was considered as standard for estimation of phenolic compounds (gallic acid). The total amount of gallic acid in ethanolic extract of *O. sanctum* leaves was observed to be 8.5 mg/g. The phytochemicals found in extract are rich antioxidants and these extracts can be used as an effective preservative in food industry.

Keywords: *ocimum Sanctum,* anti-oxidant activity, antimicrobial activity, HPLC, gallic acid

[*]**Corresponding author:** Ramesh Kumar Kushwaha
Department of Biochemistry, REVA University, Bangalore-64. Email: kush0109@yahoo.com

1. INTRODUCTION

The common name of *O. sanctum* is Tulsi. It is one of the aromatic plants, distributed mainly in the tropical and subtropical regions of the world including India. The species of *O. sanctum* is considered to be highly sacred and medicinal application. Chemical constituents with antioxidant activity present in high concentrations in plants determine their considerable role in the prevention of various degenerative diseases[1,2]. A number of phenolic compounds with strong antioxidant and antimicrobial activities have been identified in plants, especially in those belonging to the *Lamiaceae* family[3]. The genus *Ocimum,* a member of the *Lamiaceae* family, contains 200species of herbs and shrubs [4]. However, the antioxidative potential of herbs and spices is correlated with the presence of phenolic compounds in the sample. The major phenolic compounds found in plants are secondary metabolites possessing high antioxidant activity and it is widely spread in the species of *Lamiaceae*[5]. It shows medicinal property against asthma, common cold, bronchitis and respiratory tract infection. Along with being a good antioxidant activity, it also has antimicrobial and many medicinal properties. Hence an attempt has been made for identification and quantification of Gallic acid from leaves of *O. sanctum*. Presence of Gallic acid in leaves extract is determined with the aid of HPLC[6].This species have a source of aroma compounds and essential oils containing biologically active constituents that possess insecticidal and nematicidal properties[7]. The generation of reactive oxygen species (ROS) beyond the antioxidant capacity of the biological system gives rise to oxidative stress. Free radical oxidative stress has been implicated in the pathogenesis of a variety of human diseases such as atherosclerosis, diabetes mellitus, hypertension, inflammation, cancer and

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AIDS [8]. Antioxidants are a group of substances which, when present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation (as reducing agents), metal ion chelation (thereby eliminating potential free radicals), sparing of antioxidants (co-antioxidants) [9]. Antioxidants lower the burden of free radicals and they have ability to take up the free radicals and reduce the free radical and make it stable. The DPPH scavenging assay is a simple chemical experiment for the primary evaluation of any compound for its simplicity and low of cost for free radical scavenging activity[10]. Among the medicinal plants, aromatic herbs are a rich source of biologically active compounds useful in both agriculture and medicine[11]. Medicinal plants are used widely by traditional medical practitioners in their day to day practice for curing various diseases. Phytomedicine (use of medicinal herbs for treatment), for oral disorders such as dental caries and periodontal disease, has also been well practiced in traditional medicine of Indian, Egyptian, Greek, and Chinese civilizations.[12,13]. Different parts of *O.sanctum* have been used for various medicinal purposes throughout India, have been used for various medicinal purposes. Tulsi has been recognized as possessing antioxidant properties,[14] as a COX2 inhibitor,[15] and to provide protection from radiation poisoning[16] and cataracts[17]. Studies have also demonstrated anti-hyperlipidemia and cardioprotective effects of Tulsi in rats fed on a high-fat diet[18], and it is also known to promote immune system function[19]. A decoction prepared from Tulsi plant is hepatoprotective, immunomodulatory, analgesic, antipyretic, and is used as a diaphoretic in malarial fever[20]. Tulsi has also been used as an important pot herb in folklore practices for a number of ailments and diseases[21]. Tulsi also helps to prevent cancers caused by toxic compound by reducing DNA damage[22]. Inducing apoptosis in precancerous and cancerous cells, thereby reducing the growth of experimental tumors and enhancing survival [23-24].

2. MATERIALS AND METHODS

2.1 Collection of Plant Material:

Ocimum sanctum leaves were purchased from local market, Kattigenahalli, Yelahanka Bangalore, India. Leaves were dried in shade at room temperature for 7 days.

2.2 Plant Extract preparation:

5gm of leaf powder was treated with 250ml of ethanol in round bottom flask and proceeded for Soxhlet method of extraction. Temperature was fixed at 65^{o}C for 6 cycles while doing Soxhlet method of extraction. Ethanolic extract was subjected for vacuum dry at 50^{o}C to evaporate ethanol. 0.5gm of crude extract was obtained and that is dissolved in 1ml of ethanol and serially diluted to 10mg/ml.

2.3 Qualitative analysis of Secondary Metabolites.

The following preliminary test such as alkaloids, flavonoids, terpenoids, phenols and tannins as follow as below.

2.3.1 Test for Alkaloid (Mayer's test):

1ml of crude extract (10mg/ml) mixed with 1ml of Mayer's reagent in test tube and further added few drops of iodine solution in a mixture to observe the changes in color.

2.3.2 Test for Flavonoids:

1ml of 10% Lead acetate solution was treated with 100µl of plant crude extract(10mg/ml). To observe the yellow color appearance in mixture.

2.3.3 Test for Terpenoids:

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1ml of leaf extract(10mg/ml) was mixed with 1ml Conc. H_2SO_4 in test tube and then heated over the flame to observe the change in color.

2.3.4 Test for Phenols and Tannins:

1ml of leaf crude extract(10mg/ml) was mixed with 2ml of distilled water and few drops of $FeCl_3$. Mixture was subjected on magnetic stirrer for appearance of green color.

2.4 HPLC Analysis of Leaf extract:

10mg leaf extract was dissolved in 1ml of HPLC grade methanol. 20µl of extract was manually injected in HPLC (Shimadzu, water C-18-250mm×4.6mm;5µm). HPLC was performed in isocratic condition and solvent was used mixture of Acetonitrile:water:Acetic acid (15:84:1). The flow rate was fixed at 1ml/min and the chromatogram was monitored at wavelength 272nm with UV-PDA detector. The peak of standard gallic acid retention time was correlated with leaf sample chromatogram to determine the concentration of gallic acid equivalent total phenolic content.

2.5 DPPH Assay for Anti-oxidant activity

DPPH assay is carried out as per the method of Rajkumar *et al.*[25]. In brief, 80µl of DPPH solution; various concentration of test solution and quantity sufficient to 240µl with HPLC grade methanol. The different concentrations tested for REFERENCES standard are 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0 µg/ml. The different concentrations tested for test samples are 31.25, 62.5, 125, 250, 500 and 1000µg/ml. The reaction mixture is mixed and incubated at 25^{o}C for 15 minutes. The absorbance is measured at 590nm using semi-autoanalyzer. A control reaction is carried out without the test sample.

3. RESULTS AND DISCUSSION

3.1 Qualitative analysis of plant Secondary Metabolites

We performed the qualitative analysis as test for alkaloids, flavonoids, terpenoids, phenols and tannins and we found the presence of flavonoids, terpenoids, phenols and tannins, all four secondary metabolites as shown in Table 1. Edeoga et al. previously reported the presence of all four secondary metabolite (favonoids, terpenoids, phenols and tannins) in the leaf and stem of many wild plant such as Tulsi, neem, curry, amaranth, clover[26]

Table1: Preliminary test for secondary metabolites present in *O. sanctum* leaf exytract

Parameter	Result
Test for Alkaloid (Mayer's Test)	'-'
Test for Flavonoids	'+'
Test for Terpenoids	'+'
Test for Phenols and tannins	'+'

3.2 Quantification of total phenolic compound

HPLC confirmed the presence of gallic acid phenolic compound in the *O. sanctum* leaf extract. Here we calculated the TPC(Total Phenolic Content) present in leaf extract through the standard gallic acid equivalent. The total TPC was 8.5mg in 10mg of leaf extract was calculated with the REFERENCES of standards chromatogram. Figure 1 represents the chromatogram of standard gallic acid and figure 2 represents the chromatogram of *O. sanctum* leaf extract. The phenolic content is not only limited upto *O. sanctum* although has been reported from many plants like mentha, rosemary, lavender, bugle weed[5]. Here, we also found the similar observation in the leaf extract of the *O. sanctum*.

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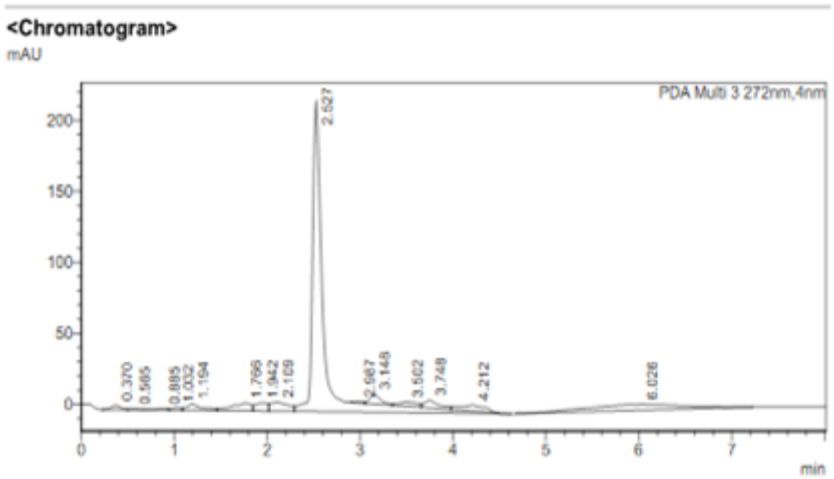

Figure 1: Chromatogram of standard Gallic acid.

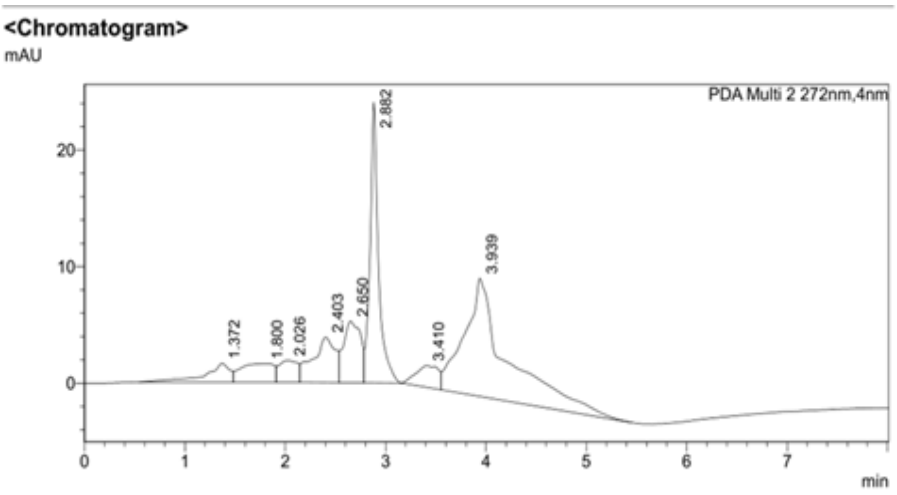

Figure 2: Chromatogram of *O. sanctum* leafextract.

3.3 DPPH Analysis

Half maximal Inhibitory concentration (IC50) is the concentration of the substance required to inhibit a biological process such as an enzyme, cell, cell receptor or microorganism by half.

Sample Code	Conc.in µg/ml	Absorbance	% Inhibition	IC50
Control	0	0.767	0	
Standard Gallic acid	0.3125	0.698	9.00	1.801
	0.625	0.619	19.30	
	1.25	0.499	34.94	
	2.5	0.364	52.54	
	5	0.258	66.36	

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	10	0.161	79.01	
Ethanol extract	31.25	0.739	3.63	863.4
	62.5	0.702	8.48	
	125	0.630	17.83	
	250	0.569	25.75	
	500	0.441	42.50	
	1000	0.283	63.10	

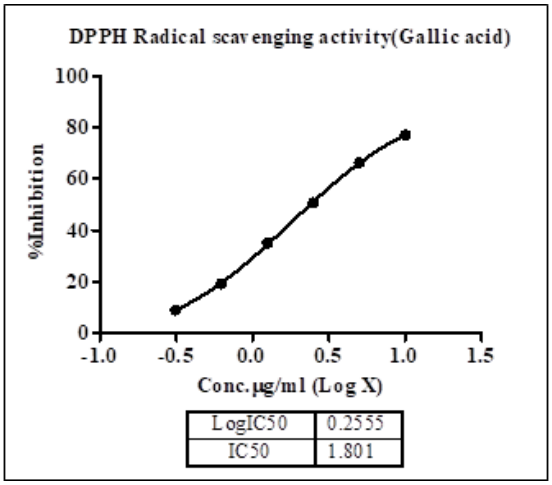

Figure-3. DPPH radical scavenging activity of Standard gallic acid

Calculating the percentage of growth inhibition:

$$\%\ Inhibition = \frac{(ODofControl - ODofSample)}{ODofControl} \times 100$$

The sample tested above showed IC50 value of 863.4µg/ml while the Gallic acid which is used as a standard showed IC50 value of 1.801µg/ml.

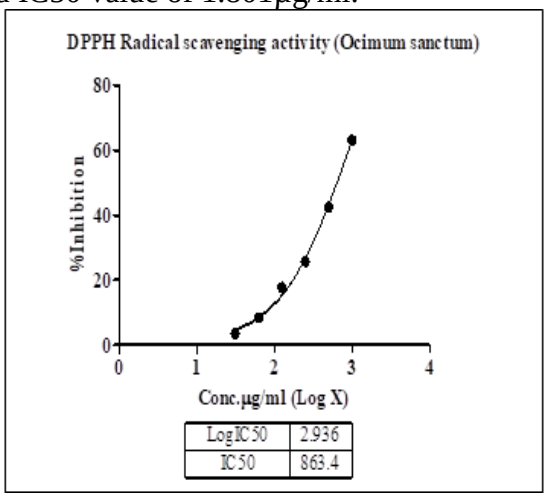

Figure-4. DPPH radical scavenging activity of *Ocimum sanctum*

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4. CONCLUSION

Here we conclude the presence of all four secondary metabolites(flavonoids, terpenoids, phenols and tannins) present in ethanolic extract of *Ocimum sanctum* leaf. HPLC analysis proves the concentration of TPC(Total Phenolic Content) was present in leaf extract. Because of TPC, *Ocimum sanctum* known for its various medicinal properties that is useful for human beings from ancient times.

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IN VITRO ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITY OF *MICHELIA CHAMPACA* LEAF EXTRACT IN RAW 264.7 CELL LINE

H N Deepa[1] , Singh Anchala[1*], U Sreenidhi[1] ,Jayashree.S[1]
[1]School of Biochemistry, REVA University, Bangalore-64, Karnataka, India

ABSTRACT: *Michelia champaca* is a commonly available tree in tropical and subtropical regions including Himalayan and Western Ghats regions of India. Since time immemorial the plant has been used in treatment of fever, leprosy, eye disorders, wound healing, inflammation, ulcer, diabetes etc. Qualitative analysis of the plant's phytochemicals have been carried out using standard procedures. It showed the presence of several phytoconstituents like alkaloids , terpenoids, phenols, tannins, flavanoids etc. This study was aimed for in-vitro assessment of anti-inflammatory activity of *M. Champaca* leaves ethanolic extract in RAW 264.7 Cell line. Ferulic acid is a Phenolic compound known for its various therapeutic properties such as anti-inflammation, antioxidant, antipyretic, antihelminthic, antimicrobial etc. The extract was quantified for concentration of Ferulic acid by RP-HPLC. The anti-inflammatory activity of leaf extract was determined through nitric oxide scavenging Assay. IC_{50} value of ethanolic extract of *M. champaca* leaves was found to be 212.81 µg/ml. As it is less toxic and gets metabolised and absorbed easily, Ferulic acid can be recommended as a compound against inflammatory diseases with great potential.

KEYWORDS: Ferulic acid, Anti-inflammatory, RP-HPLC, Nitric Oxide Scavenging Assay, RAW 264.7 Cell line

[*]**Corresponding author: H N DEEPA**
REVA University, School of Biochemistry, Bangalore-64, Karnataka, India
Email: deepa.hn@reva.edu.in

1. INTRODUCTION

Inflammation is a response against cell or tissue injury which paves pathway for healing a wound by proceeding a chain of reactions which trigger cell repairing mechanism and reconstitute the site of injury. It is body's defense mechanism which comprises of complex biochemical reactions and hence limits the spread of infectious and injurious agents followed by elimination of damaged tissues. Inflammation is caused by infective agents (pathogens), immunological agents (drugs), physical agents (environmental factors), chemical agents , inert substances etc [1] . Inflammation is of two types- Acute and Chronic. Acute inflammation shows immediate response against injuries and it is of short duration. Chronic inflammation occurs due to long persisting acute inflammatory responses. Wound healing takes long time in case of chronic response [2].

Cell lines are used for *in vitro* and *in vivo* study and analysis. Unbound sources for cell lines with similar genotypic and phenotypic character can be obtained through serial passaging [3]. The RAW 264.7 cells are macrophage cells, which originates from Abelson leukemia virus transformed cell line derived from BALB/c mice. These cells can perform pinocytosis and phagocytosis. Lipopolysaccharide (LPS) is found as a major component of the cell wall of gram-negative bacteria. LPS stimulates immune cells to induce inflammatory responses and causes the production of pro-inflammatory cytokines [4]. Production of Nitric Oxide (NO) increases when cells get stimulated by lipopolysaccharide. It also enhances phagocytic nature of cell. Nitric oxide (NO) shows the degree of LPS-induced inflammation in macrophages

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[5]. During the inflammatory process, NO is produced by nitric oxide synthase (iNOS), which plays a regulatory role in the expression of pro-inflammatory mediators. Furthermore, these cells are able to kill target cells by antibody dependent cytotoxicity [6].

Therapeutic usage of parts of *M. champaca* are known since time immemorial. The plant, commonly known as *Champaca*, is native to India and distributed in Indo-Malaysian region, Eastern Himalayan region and Western Ghats of India [7]. Previously it has been reported that *champaca* has been used for treating fever [8], inflammation [9], helminthiasis [10], tumour [11], diabetes [12] , oxidative stress [13], ulcer [14] , wound [15] etc.

A growing trend for reviving Ethno-medicine projects plants as compatible sources for anti-inflammatory compounds [16][17]. The preliminary phytochemical screening and TLC fingerprinting of whole plant extracts of *M. champaca* has been done and benchmarked with presence of various phytoconstituents[18].

Nitric oxide (NO) is a short-lived free radical that regulates inflammation. It enhances bactericidal activity of activated Macrophages. But excess production of Nitric Oxide causes tissue damage and thus activates pro-inflammatory mediators. The potential of extracts from medicinal plants to scavenge these free radicals and modulate inflammatory reactions has been demonstrated [19].

2. MATERIALS AND METHODS

2.1 Collection of Plant Sample

Leaves of *M. champa*ca were procured from the area of Kattigenahalli, Bangalore. The collected leaf sample was identified and authenticated by University of Agriculture Sciences , GKVK, Bangalore.

2.2 Preparation of Plant Extract

Leaves of *M. champaca* were dried for 15 days under dark. Later the sample was powdered using Grinder and sieved for removing coarse particles. Total quantity of fine powder was 16.0 g. It was subjected to soxhlet extraction using absolute ethanol as solvent[20]. A packet of blotting paper was made and filled with powdered leaf sample. The packet was placed in thimble of soxhlet extractor and operated for a period of 3.5 hrs at the range of 65°C ± 2°C. A total of 6 cycles were performed each spanning about 30-40 mins.

The ethanolic extract was obtained and subjected to further evaporation of extra solvent using rotavaps operated for 30-35 mins at 50°C. The solvent was removed under vacuum. The dried crude extract was weighed to be 2.0g[21].

2.3 Preliminary Test for Phytochemicals

Dried crude ethanolic extract was used for screening the phytochemical presence in *M. champaca* leaf [18]. The procedure was carried out using Standard qualitative methods as follows:-

2.3.1 Test for Alkaloid

This test was carried out using Mayer's test.1mg of crude extract was taken in a test tube and 0.2 ml of Dil. HCl and 0.1 ml of Mayer's chemical agent were added.

2.3.2 Test for Phenols and Tannins

1.0 mg of crude extract was mixed in 2.0 mL of distilled water taken in test tube. 3-4 drops of $FeCl_3$ Solution were added and observed for the formation of precipitate.

2.3.3 Test for Flavonoids

1.0 mg of crude extract was mixed with 1.0 mL of 10% lead acetate solution.

2.3.4 Test for Terpenoids

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1.0 mg of crude extract was mixed with 2 ml of Chloroform and evaporated to make it dry. 2 ml of Conc. H_2SO_4 was added and heated for about 2 min.

2.4 Estimation of Ferulic acid

The instrument used was an RP-HPLC, Shimadzu Corp., Kyoto. It consists of a LC-20 AD Solvent delivery pump, Prominence system controller CBM 20A Lite, Prominence Photodiode Array detector SPD-M20A and a manual injector with 100µl syringe and adaptar plate. The system was incorporated with LabSol LC Connect CS Ready software.

2.4.1 Preparation of Standard Ferulic acid Solution :

1 mg of Ferulic acid (98% purity, purchased from HiMedia) was dissolved in 1 ml of HPLC grade methanol.

2.4.2 Preparation of plant sample

1mg of the dried crude extract was dissolved in 1ml of HPLC grade methanol.

2.4.3 HPLC Analysis:

Reverse-phase chromatographic analysis was carried out in isocratic condition using a C-18 analytical column (250×4.6 mm, 5µm) at 25°C. The gradient elution of solvent A (Distilled water- 140 ml) and solvent B (Acetonitrile-Acetic acid- 3:1) had a significant effect on the resolution of compounds. As a result, solvent gradient was formed by using dual pumping system which causes variation in the proportion of solvent A to solvent B. The flow rate was adjusted to 1.0 ml/min and detection wavelength was setup at 254 nm. 20µl of standard Ferulic acid was manually injected into HPLC and run time was set for 25 mins. The peak area was calculated by using incorporated software. Then 20µl of plant sample was injected into HPLC and run time was set for 25 minutes. The peak area was calculated by using software [22].

2.5 Nitric Oxide Scavenging Assay

2.5.1 Cell Culture

The murine monocytic macrophage RAW 264.7 cell line was cultured in Dulbecco's Modified Eagle Media (DMEM) (2 mM L-Glutamine, 45 g/l Glucose, 1 mM Sodium Pyruvate) with 10% Fetal Bovine Serum (FBS) at 37°C with 5% CO_2 and were subcultured twice a week. The cells were then seeded in 96-well tissue culture plates (1×10^6 cells/ml) and incubated for 24 hrs at 37°C with 5% CO_2 [3].

2.5.2 Preparation of test extract

100 µl of plant extract in DMSO was serially diluted to give a two-fold concentration variations and then added into wells of cell culture plate which is pre-incubated with cells. Cells were then stimulated by adding 10 µg/ml *Escherichia coli* Lipopolysaccharide (LPS) and incubated at 37°C for another 17 hrs. After 24 hrs incubation, 100 µl of supernatant from each well of cell culture plate was transferred into 96-well micro-titre plate and equal volume of Griess reagent was added. The absorbance of the resultant solutions in wells of the micro-titre plate was determined with a micro-titre plate reader (Tecan device) at 550 nm after 10 minutes of incubation [23], [24].

3. RESULTS

3.1 Preliminary Test for Phytochemicals

M. champaca ethanolic leaf extract was subjected for the secondary metabolite tests to check the presence of alkaloids, phenols, tannins, flavanoids and terpenoids. Different secondary metabolite gives different colour according to the procedure (figure 1). Reddish brown colour indicated the presence of alkaloids. Formation of green colour precipitate indicated the

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presence of phenols and tannins. Yellow and grey colour represented the presence of flavanoids and terpenoids respectively in the leaf extract of *M. champaca*. Their results are depicted in table 1. The colour indication of secondary metabolites are shown in figure 1.

Table 1: Result of Preliminary Tests for Phytochemicals

Experiment	**Observation**	**Inference**
Test for Alkaloids	Reddish brown colour precipitate was formed	Alkaloids are present
Test for Phenols and Tannins	Green colour precipitate was formed	Phenols and Tannins are present
Test for Flavanoids	Yellow colour precipitate was formed	Flavanoids are present
Test for Terpenoids	Grey colour precipitate was formed	Terpenoids are present

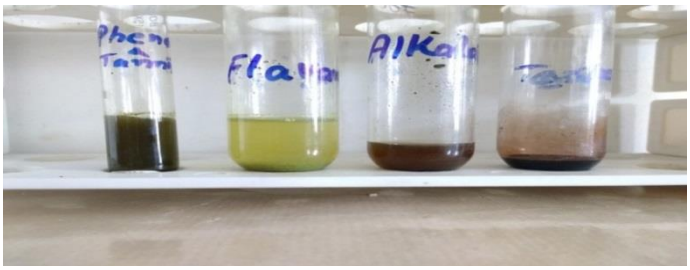

Figure 1. Result for Preliminary Test for Phytochemicals

3.3 Estimation of Ferulic acid:

The concentration of Ferulic acid in *M. champaca* leaf extract was estimated by using RP-HPLC method. The concentration of Ferulic acid was found to be 2.3 µgof Ferulic acid in 1 mg of leaf crude extract. Quantitative analysis of Ferulic acid in the crude plant extract was obtained by using the following formula:

$$\frac{\text{Concentrationof Standard FerulicAcidsolution}}{\text{AreaofPeak}} = \frac{\text{Concentration of Ferulic Acid in leaf extract}}{\text{Area of Peak}}$$

HPLC profile of Standard Ferulic acid Solution and Crude plant extract has been represented in figure 2 and Table 2; figure 3 and Table 3 along with their retention time respectively.

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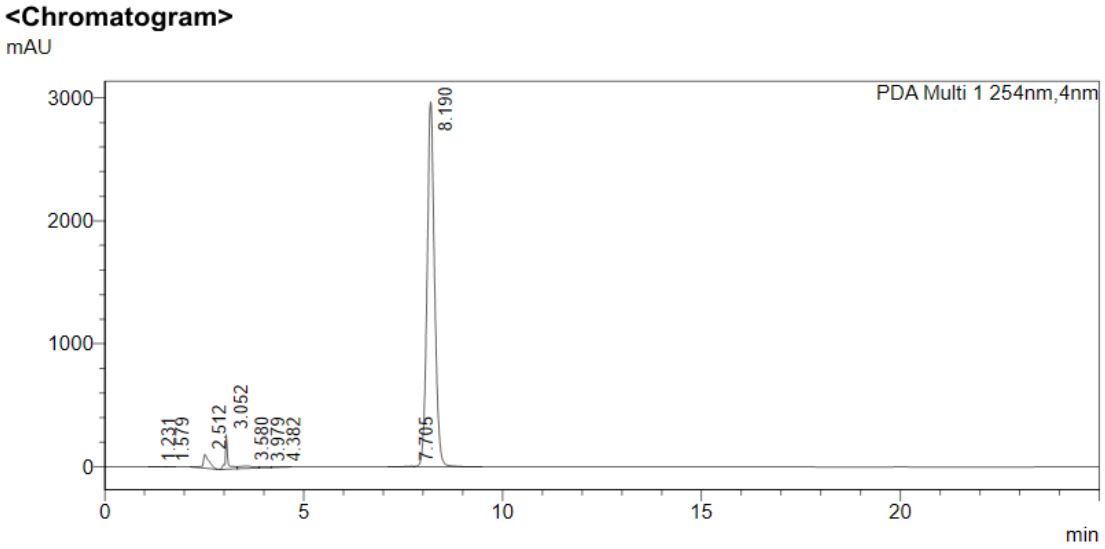

Figure 2. RP-HPLC Analysis of Ferulic acid Standard

Table 2: RP-HPLC analysis of Ferulic acid standard

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height
1	1.231	8103	682
2	1.579	2605	281
3	2.512	1168591	110325
4	3.052	1216832	282350
5	3.580	497167	18811
6	3.979	137715	8463
7	4.382	91889	3786
8	7.705	46188	3238
9	8.190	38939548	2967005
Total		42108637	3394940

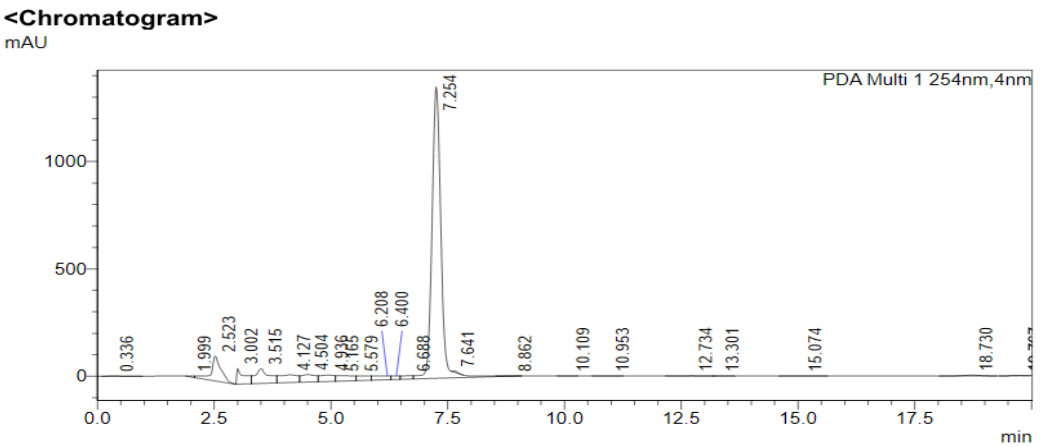

Figure 3: RP HPLC Analysis of Leaf Extract of *M champaca*

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Table 3: RP HPLC Analysis of Leaf Extract of *M champaca*

PDA Ch1 254nm

Peak#	Ret. Time	Area	Height
1	0.336	5806	212
2	1.999	35585	3685
3	2.523	1650950	114917
4	3.002	844241	72104
5	3.515	1408976	67206
6	4.127	987765	36254
7	4.504	749490	35177
8	4.936	634268	29476
9	5.165	639040	25969
10	5.579	418223	22197
11	6.208	465837	17995
12	6.400	207218	16978
13	6.688	267919	15977
14	7.254	18195033	1358115
15	7.641	41493	5009
16	8.862	3446	222
17	10.109	1854	140
18	10.953	5476	256
19	12.734	36893	992
20	13.301	4878	350
21	15.074	23344	894
22	18.730	91828	3001
23	19.767	19917	921
Total		26739479	1828049

Concentration of the compound:

Concentration of Standard Ferulic acid Solution = 1000µg/mL

Calculation for Concentration of Ferulic acid :

$$\Rightarrow \frac{\text{Concentrationof Standard FerulicAcidsolution}}{\text{AreaofPeak}} = \frac{\text{Concentration of Ferulic Acid in leaf extract}}{\text{Area of Peak}}$$

Hence 1mg of leaf extract contains 2.3 µg of Ferulic acid.

3.4 Nitric Oxide Scavenging Assay:

The concentration of nitrite was calculated from Regression analysis using serial dilutions of Sodium Nitrite as a standard. Percentage inhibition was calculated based on the ability of extract to inhibit Nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0 % inhibition.

Table 6: Absorbance at 550 nm

Compound name	Concentration (µg/ml)	OD at 590nm	% Inhibition	IC_{50} (µg/mL)
Control	0	0.7551	0.00	
Michelia champaca	10	0.6978	7.59	
	20	0.6279	16.85	
	40	0.5927	21.51	212.81
	80	0.5294	29.89	
	160	0.4186	44.57	
	320	0.2916	61.38	

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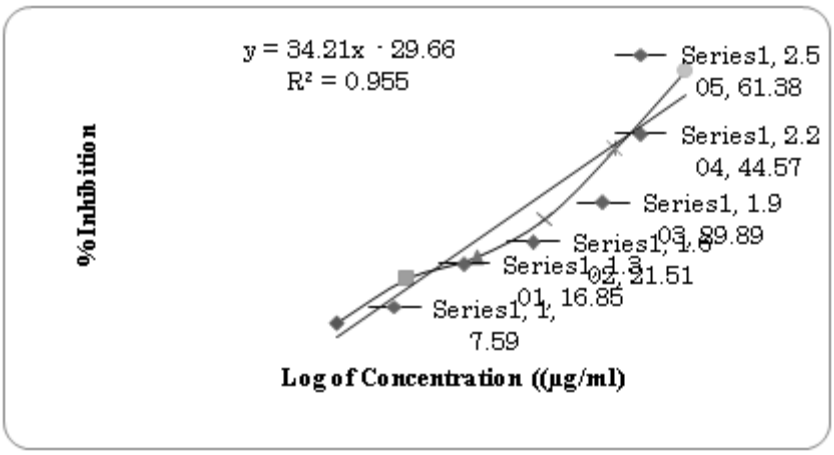

Figure 4. Graphical representation of log of concentration vs. % Inhibition

3.5 Determination of IC_{50} value:

The linear equation of the above graph is y=34.218x-29.669 ─ eq. (1)
For IC_{50} value, y = 50
Therefore, 50 = 34.218x-29.669 ─ eq. (2)
Solving the eq. (2), Value of x= 2.328
As IC_{50} = Anti log of x = Anti log (2.328) =212.81
Hence the IC_{50} value of *M. champaca* leaf extract is 212.81 µg/ml.

4. DISCUSSION

Medicinal values of plants are mainly determined by its phytochemicals.Positive results for phytochemicals like alkaloids, phenols, tannins, flavanoids and terpenoids suggests a good response for therapeutic usage of *M. champaca*[25]. Ferulic acid was estimated by RP-HPLC method. Previously done work has shown retention time of Ferulic acid at 8.0 min [26]. The retention time for standard ferulic acid was found to be 8.1 min. A good concentration of Ferulic acid (2.3 µg FA/mg extract) was found in leaf extract. Upon biological testing of the plant sample on Cell line, proper inference about its action can be well analysed and understood. Nitric Oxide scavenging assay was done on RAW 264.7 Cell line to check its inhibitory activity [27]. IC_{50} value is the concentration at which the test sample shows 50% inhibition. The IC_{50} value of extract was found to be 212.81 µg/ml. Hence, *M.champaca* leaf extract which contains Ferulic acid can be recommended as a plant with therapeutic properties against inflammatory diseases

5. CONCLUSION

This research analyzes phytochemicals of leaf ethanolic extract of *M. champac*a followed by estimation of Ferulic acid. Ferulic acid in leaf extract presented it as an efficient medicinal plant. Further testing of leaf ethanolic extract on RAW 264.7 cell line concluded it as an efficient source for scavenging Nitric oxide. Nitric oxide plays an important role in regulating inflammatory response . Thus inhibiting its production can treat inflammatory diseases. It concludes its Nitric oxide scavenging property. Thus leaves of *M. champaca*

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may be used for the treatment of inflammatory diseases.

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CONFLICT OF INTEREST

None.

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BIOSORPTION AND DECOLORIZATION OF SYNTHETIC DYES USING *SPIRULINAPLANTENSIS*

P.G. Priyadarshini[1], Sahana Rao[1]
[1]Department of life science, Garden City University, Bengaluru-560049

ABSTRACT: Synthetic dyes are the intractable substances which are one of the main residues found in the effluvium of the textile dye industries. The effluent which are released in to the environment by the industry and the domestic activity affect the environment and have impact on plant, animal, humans and aquatic habitat. The exposure of dye in the open atmosphere for a longer time leads to the formation of toxic elements which have an impact on biota. Contaminated water from the industry is produced during dyeing process. In many cases, wastewater is then a consortium of various dyes that did not affix to the fabrics during the dyeing processes. If this is untreated and discharge from textile plants into surface waters which alters the dynamics within the stream, producing unwanted pollution, increased toxicity, and eutrophication. In order to overcome this problem, bioremediation of the dyes is possible using naturally occurring micro flora, among which Cyanobacteria is predominant. *Spirulina*is an effect organism among Cyanobacteria which has a wide range of application in the field ofmedicine, medical biotechnology, environmental biotechnology because of its specialized characters such as it is economical; it is accessible, eco-friendly, nutrition and its metabolic activity. Because of these advanced features of *Spirulina*, it is being used for decolorizing syntheticdyes.

Keywords: - Dyes, Cyanobacteria, *Spirulina*, Decolorization, Biosorption, Ecosystem

***Corresponding author:** SahanaRao (Ph. D.)
Department of Lifesciences, Garden City University, Bangalore Karnataka India.
Email address: sahana.rao@gardencity.university

1. INTRODUCTION

Synthetic dyes are intractable substances molecules that constitute the main remnant part found in the in the effluvium of the textile dye industries. [1] The dyes are generally unadoptable to biodegradation due to their xenobiotic nature. [1] In some textile dye industries 15-20% of the dyes used don't affix to the fibers as a result of this they are being let into water resources causing water pollution [2]. Dyeing waste which are let released into water bodies by the activity of textile industries is been reported as a threat to environment. [3]. In 21st century synthetic dyes were completely dominated the textile industries and paper industries because of its better color fastness and less expensive, 1000 of distinct synthetic dye have been produced in the world. Over 10,000 dyes with an annual production over 7-105 metric tons worldwide are commercially available and 5-10% of the dyestuffs are lost in industrial effluents. [4]. There are some reports on toxicity and genotoxicity of textile dyes [4]. If untreated, discharge from textile plants into surface waters alters the dynamics within the stream, producing unwanted pollution, increased toxicity, and eutrophication. The discharge of these dyes into the water resources leads to the blocking of sunlight to the water bodies. Which leads to decrease in the photosynthesis activity and decrease in oxygen level; this condition leads to killing

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the marine organisms. [5] The exposure of dye in the open atmosphere for a longer time leads to the formation of toxic elements which have an impact on biota. In order to overcome this problem, the conventional methods which are used to decolorize dyes are electro coagulation, chemical coagulation, oxidation and so on, the chemicals used to for these processes are alum, ferric chloride and ferric sulphateetc., these chemicals cause harmful effects on human health and also to the environment such as wheezing, diarrhea, neurodegenerative disease and sometimes it is poisonous to the aquatic animals. In order to solve this problem, bioremediation of the dyes is possible using naturally occurring micro flora, among which Cyanobacteria is predominant. *Spirulina*is an effect organism among Cyanobacteria which has a wide range of application in the field of, medicine, medical biotechnology, environmental biotechnology because of its advance Specialized characters such as economical, it is accessible, eco-friendly, nutrition and its metabolic activity. Because of this advanced feature of *spirulina*, it is used for Decolorization and bioremediation synthetic dyes.

2. MATERIALS AND METHODS

2.1. To obtain pure culture of *Spirulinaplantensis*

The pure cultures were obtained from GKVK, Bangalore; Zarrouk's media is used for culturing *spirulina*. The growth phase of *spirulina*is 14 days at room temperature. After the growth, they are used for bio-absorption of synthetic dyes.

2.2 Chemical Modification of *Spirulinaplantenisis*

2.2.1 Chemical modification of *spirulina*with potassium permanganate ($KMNO_4$)

1. Prepare 1M KMNO4 (weigh 158.034g in 1000ml of distilled water).
2. Weigh 4 grams of *spirulina*biomass and add this to prepared 1M $KMNO_4$ solution.
3. Incubate the solution in shaker incubator for 24hrs
4. After incubation the solution is centrifuged at 1000rpm for 10 minutes
5. The pellet was collected and the supernatant were discarded
6. The collected pellet was transferred onto the aluminum foil.
7. The pellets were dried in hot air oven at 65^0C for 3 hours
8. After drying the powder were collected and stored in an air tight glass bottle.
9. The collected powder then used for Bio absorption process.

2.2.2 Chemical Modification of *spirulina*with Hydrochloric acid (HCl)

1. Prepare 1M Hydrochloric acid (36.46ml in 1000ml of distilled water) .
2. Weigh 4 gram of *spirulina*raw biomass and to the prepared 1M HCl solution
3. Incubate the solution in shaker incubator for 24hrs
4. After incubation the solution is centrifuged at 1000rpm for 10 minutes
5. The pellet was collected and the supernatant were discarded
6. The collected pellet was transferred onto the aluminum foil.
7. The pellets were dried in hot air oven at 65^0C for 3 hours
8. After drying the powder were collected and stored in an air tight glass bottle.
9. The collected powder then used for Bio absorption process.

2.2.3 Chemical Modification of *spirulina*with Nitric acid (HNO3)

1. Prepare 1M nitric acid (63.01ml in 1000ml of distilled water)
2. Weigh 4 gram of *spirulina*raw biomass and to the prepared 1M HNO_3 solution

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3. Incubate the solution in shaker incubator for 24hrs
4. After incubation the solution is centrifuged at 1000rpm for 10 minutes
5. The pellet was collected and the supernatant were discarded
6. The collected pellet was transferred onto the aluminum foil.
7. The pellets were dried in hot air oven at 65^0C for 3 hours
8. After drying the powder were collected and stored in an air tight glass bottle.
9. The collected powder then used for Bio absorption process.

2.3 Biosorption Of Synthetics Dyes

2.3.1 Preparation of dye solution

Crystal Violet- 2% solution (2grams in 100ml of distilled water)
Malachite Green- 2% solution (2grams in 100ml of distilled water)
Methyl Red- 2% solution (2grams in 100ml of distilled water)

2.3.2 Biosorption of Crystal Violet

1. To 20ml conical flask add 18ml of distilled water add 2ml of 2% crystal violet dye solution and add 2 grams raw biomass of spirulina.
2. To 20ml conical flask add 18ml of distilled water add 2ml of 2% crystal violet dye solution and add 2 grams of chemically modified *spirulina* with potassium permanganate.
3. To 20ml conical flask add 18ml of distilled water add 2ml of 2% crystal violet dye solution and add 2 grams of chemically modified *spirulina*with nitric acid.
4. To 20ml conical flask add 18ml of distilled water add 2ml of 2% crystal violet dye solution and add 2 grams of chemically modified *spirulina* with hydrochloric acid.

2.3.3 Biosorption of Methyl Red

1. To 20ml conical flask add 18ml of distilled water add 2ml of 2% methyl red dye solution and add 2 grams raw biomass of spirulina.
2. To 20ml conical flask add 18ml of distilled water add 2ml of 2% methyl red dye solution and add 2 grams of chemically modified *spirulina* with potassium permanganate.
3. To 20ml conical flask add 18ml of distilled water add 2ml of 2% methyl red dye solution and add 2 grams of chemically modified *spirulina*with nitric acid.
4. To 20ml conical flask add 18ml of distilled water add 2ml of 2% methyl red dye solution and add 2 grams of chemically modified *spirulina* with hydrochloric acid.

2.3.4 Biosorption of Malachite Green

1. To 20ml conical flask add 18ml of distilled water add 2ml of 2% Malachite Green solution and add 2 grams raw biomass of spirulina.
2. To 20ml conical flask add 18ml of distilled water add 2ml of 2% Malachite Green solution and add 2 grams of chemically modified *spirulina* with potassium permanganate.
3. To 20ml conical flask add 18ml of distilled water add 2ml of 2% Malachite Green solution and add 2 grams of chemically modified *spirulina*with nitric acid.
4. To 20ml conical flask add 18ml of distilled water add 2ml of 2% Malachite Green solution and add 2 grams of chemically modified *spirulina*w ith hydrochloric acid.

The following conical flasks were incubated for 14 days at room temperature followed by

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this the Decolorization of the dyes was analyzed by UV-Visible Spectrophotometer.

3. RESULTS

3.1 Results UV-Visible Spectrophotometer of The Decolorization Of Dye

The chemical modification of *spirulina* is done using KMNO4, HNO_3 and HCl. In order to increase the ability of *spirulina* for absorbing the synthetics dye. The chemical modification helps in opening the membrane channels of *spirulina*, which will help in the bio absorption process.

KMNO4 serves as an oxidizing agent, HNO_3, HCl serves an acidifying agent.

UV-Visible spectrophotometer analysis of crystal violet, methyl red and malachite green shown in Table 1. The readings of Raw , $KMNO_4$, $HNO_3$and HCl are lesser than the standard and show decolourization. Fig 2(a) and 2(b) The Decolorization range was statistically analyzed.

After obtaining the absorbance values, a graph is plotted. (Fig 1)

Table 1: The following readings of test solutions without dilution

Dye name	Standard	Raw	KmnO4	HNO_3	HCl
Crystal violet	1.48	1.38	1.06	0.11	0.02
Methyl red	1.20	0.95	0.70	0.26	0.24
Malachite green	1.73	1.09	1.44	0.09	0.05
Mean	1.47± 0.15	1.14±012	1.06±0.2	0.15±0.05	0.10±0 .06

Statistical analysis:The results are expressed as the mean ± standard error (SE). A descriptive statistical analysis was used to evaluate the data with the significance level of $P< 0.05$.

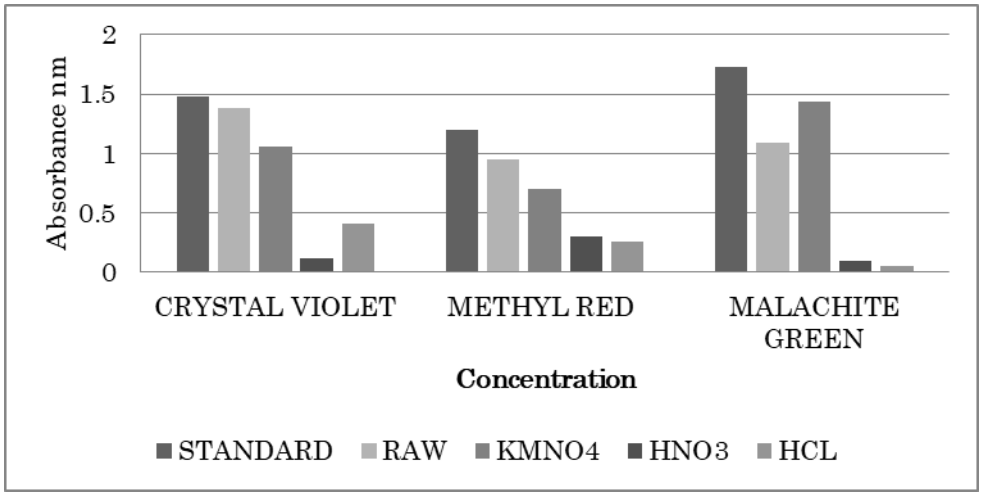

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Fig 1. By the above graph we can see that the peak of standard is higher than all other peaks, which symbolically depicts the Decolorization of dyes.

Fig 2. (a) Untreated dye solution Fig 2. (b) Treated dye solution

3.2 SEM results of the raw biomass and modified *spirulina* using $KMNO_4$,HCl and HNO_3.

There is structural modification in chemically modified *spirulina* and raw biomass. The images of SEM show that all the images are different from one another. (fig 2,3,4 &5)
All the images are scanned and viewed under 10µM and 5µM. The structural medication can be identified by looking at the images.

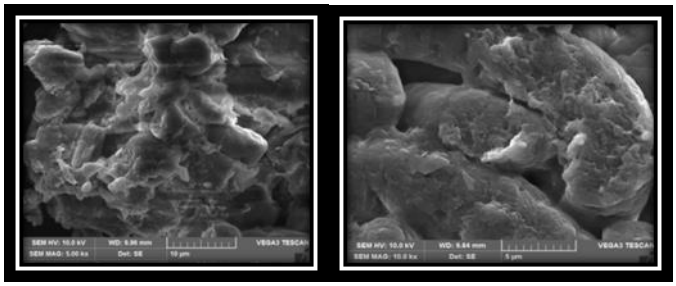

Fig 3 Fig 4

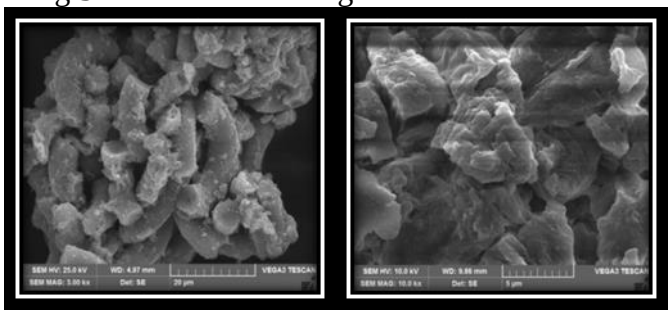

Fig-5 Fig-6

Fig 3,4, 5 and 6: represent the SEM images of raw biomass of *spirulina*, chemically modified *spirulina* using potassium permanganate, hydrochloric acid and nitric acid respectively.

3.3 The Results of FTIR Graph interpretation of Raw biomass and chemically modified *Spirulina*

Firstlythe range from 4000 to 2500 wave length in the x axis the highest peak depicts the presence of functional group. When comparing to the standard graph the remaining three graph are showing different range of peaks Standard- 2923cm^{-1}, 3282cm^{-1}, *Spirulina*

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modified with HCl- 3271cm^{-1}, with HNO_3 – 3291cm^{-1} with KMNO4 – 3175cm^{-1}.

By the graph we conclude that there is deviation in the peak when compared to standard graph, therefore we can conclude that there is modification in the functional group present in modified *spirulina*

Secondly the range from 2500 to 2000 is the region where the presence of triple bds.We can observe the deviation in the peak when compared to standard graph.Standard- 1531cm^{-1}, 1629cm^{-1}, HCl – 1637cm^{-1} , HNO_3- 1621cm^{-1} ,KMNO4- 1504cm^{-1}.

Thirdly the range from 2000-1500 there are many peaks, this is the region of identity it is unique for every component.

Finally, by observing the graph we can conclude that there is deviation in the peaks at all three regions.

The FTIR graph was analyzed by the standard values.

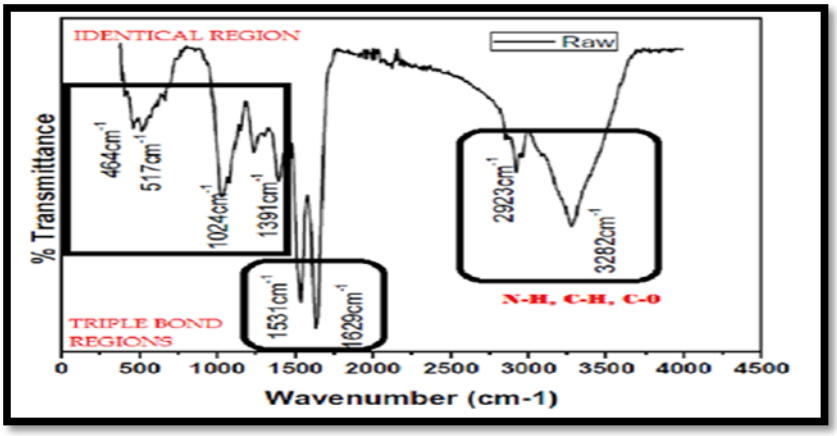

Fig 7. The FTIR graph interpretation of raw biomass and chemically modified *spirulina*

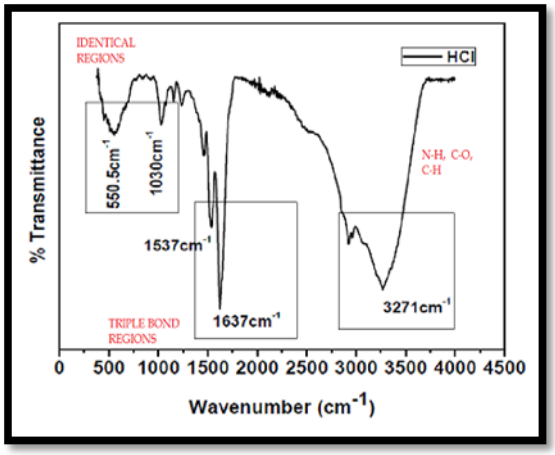

Fig: 8

Modified *Spirulina* using HCl

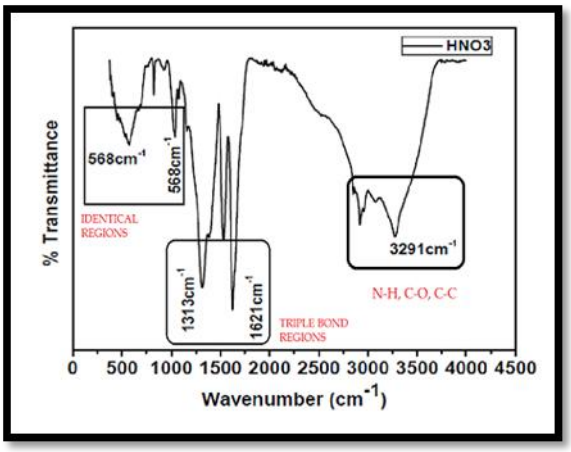

Fig: 9

Modified *Spirulina* using HNO_3

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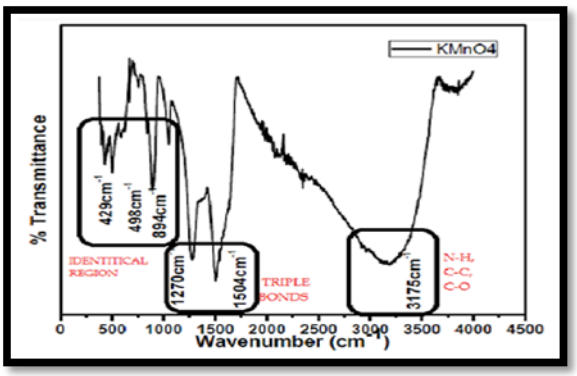

Fig 10- Modified *Spirulina* using KMNO4

3.4 Comparative study of the Decolorization between methyl red, malachite green andCrystal violet

By the UV visible spectrophotometer analysis we can conclude that *spirulina plantensis* has more effectively decolourized malachite green more than methyl red and crystal violet.

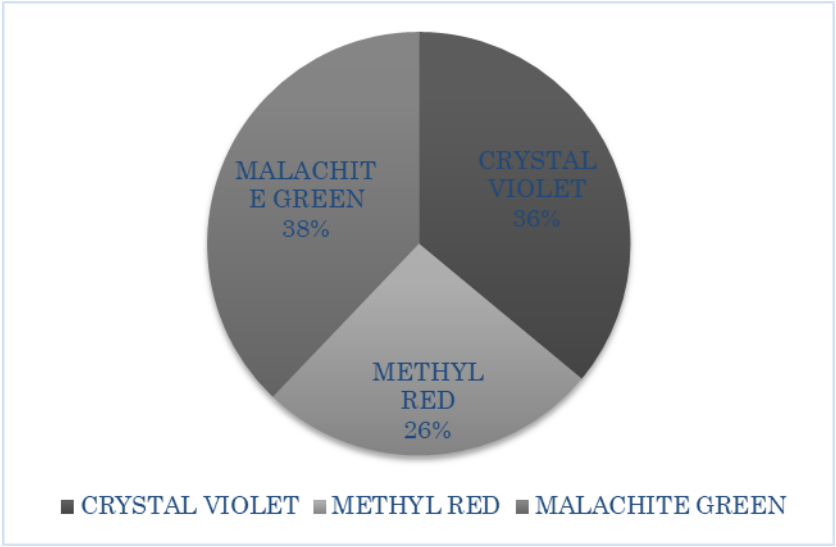

Fig 10. UV-Visible spectrophotometer analysis for*Spirulinaplantensis* has more effective decolorized malachite green more when compared to methyl red and crystal violet.

4. DISCUSSION

Bioremediation is an innovative technique that uses algae to clean up untreated industrial discharges. It is based on the advantage of the alga's natural ability to take up, accumulate and degrade the constituents that are present in their growth environment. [8] cyanobacteria induced decolorization and detoxification of the effluvium was observed in chemically modified *spirulina* using potassium permanganate, nitric acid and sulphuric acid. Similar results were observed in Cyanobacteria-induced decolorization and detoxification of Effluvium was analyzed for three cyanobacteria species. Which decolorized palanil yellow, indanthrenered and indigo dye. [10] The results have showed that cyanobacteria were able

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to reduce the pollutant content of the effluent. [2]

The potential of Cyanobacteria sp *spirulina* has ability to decolorize synthetic dyes. Our work was based on decolorizing synthetic dyes such as malachite green, methyl red and crystal violet using *spirulina* gave positive results by observing in UV spectrophotometry readings whichshowed peaks at 460nm. Similar results were seen in the blue dye treated *spirogyra* sp. which showed peaks at 736, 615 and 720nm. [1]

FTIR analysis of the chemically modified *spirulina* comparing with raw biomass showed positive results. In the first region, from 4000 to 2500 wave length in the x axis, Standard-2923cm^{-1}, 3282cm^{-1}, *Spirulina* modified with HCl- 3271cm^{-1}, *spirulina* modified with HNo_3 – 3291cm^{-1} and modified with KMNO4 – 3175cm^{-1}.similar results were seen with the FIR spectra of blue parent dye which displayed peaks at 3316, 2118, 1637, 578, 552, 504, 564, 534, 524,505,522,524 and 508cm^{-1}.[1]

The SEM analysis of chemically modified *spirulina* comparing with raw biomass showed positive results. When compared to other conventional methods which involve the use of enzymes and chemicals. [6] Bioremediation of dyes using *spirulina* is beneficial when compared to microbes because *spirulina* does not induce toxicity when compared to microbes. [2][4][5]. Finally, we can conclude *spirulina* is predominant organism which can effectively use for Biosorption and Decolorization of synthetic dyes.

5. CONCLUSION

The present work is based on the Decolorization and the Biosorption of textiles dyes using *spirulina*. The *spirulina* (raw biomass) were treated with chemicals such as potassium permanganate, hydrochloric acid and nitric acid, where potassium permanganate serves as an oxidizing agent, hydrochloric acid and nitric acid serves as an acidifying agent. Chemical modification of *spirulina*is done to increase the efficiency of *spirulina* to decolorize the dyes. The FTIR and SEM results of the chemically modified *spirulina* showed positive results in their chemical properties and also in their structure. The Decolorization of crystal violet, malachite green and methyl red were done using raw biomass and chemically modified *spirulina* with potassium permanganate, hydrochloric acid and nitric acid. The Decolorization was examined using UV Visible spectrophotometer. The results of UV Visible spectrophotometer showed positive results for Decolorization of malachite green, crystal violet and methyl red. The comparative analysis of Decolorization of dyes showed that malachite green showed high rate Decolorization than methyl red and crystal violet. Therefore, we can conclude that *spirulina* has effectively decolorized malachite green, methyl red and crystal violet.

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QUALITATIVE ANALYSIS OF PHYTOCHEMICAL COMPOUNDS FROM THREE TYPES OF RAISINS AND DETERMINATION OF THE QUANTITY OF GALIC ACID WITH THE HELP OF HPLC

Jayasri.P[1*], Rajesh Das[1], Tamosha Mitra[1], Debasmita Bhadury[1],
[1]Department of Biochemistry, Reva University, Bangalore

ABSTRACT: Dried fruits contain a high amount of energy and nutrient density compared to the fresh ones. Raisins contain antioxidants which add to their potential health benefits. This paper presents a broad insight into the phytochemicals present in three different types of Raisins. The bioactive property of phytochemicals points to their ability as natural drug products to thwart and treat human ailments. Compounds with antioxidant properties may symbolize a novel set of safe and efficient drugs. The phytochemicals from Raisins were extracted by using an orbital shaker and rotatory evaporator, followed by qualitative tests for alkaloids, terpenoids, tannins, and phenols. Gallic acid is a phenolic acid which is known to show antihyperlipidemic, antihyperglycemic, antioxidant and anticancer effects. Hence, in our study, the quantity of gallic acid in three types of Raisins was determined by High-Performance Liquid Chromatography. The result revealed that golden raisins contain more amount of Gallic acid. Our investigations form the basis of health benefits associated with Golden Raisins.

KEYWORDS:Gallic acid, Raisins, Phytochemical.

[*]Corresponding authors: Jayasri.P
Email: jayasri.p@reva.edu.in
Mob No. 9902402820

1. INTRODUCTION

Among all the dry fruits raisins rank the highest in the concentration of total phenolic compounds and antioxidant activities among solid fruit products. After the U.S. and Turkey, China is the world's third largest producer of raisins. Most grapes are grown in Xinjiang province, China's largest province, spanning an area which combines the size of France, Spain, Italy, and Germany or the six western states of the USA[1]. Polyphenols are one of the examples of plant secondary metabolites[2]. Low moisture content in raisins increases its polyphenol content[3].

According to the Food and Agriculture Organization (FAO), globally the grape production reached in 2013 was 75 million tons and had a monetary value of US$55 million.[4]. Grape is such a fruit that grows on the perennial and deciduous woody vines of the genus *Vitis*[5]. Raisins obtain their dark brown coloring from an accumulation of brown-black melanin pigments produced by polyphenol oxidase activity and no enzymatic reactions[6]. Fructans in raisins have previously been reported by a single commercial laboratory [7].

The skin of some fruits such as grapes is covered by a waxy coating that reduces permeability and therefore hinders the loss of water. That is why before artificial drying other chemical and physical pre-treatments are used to enhance permeability by increasing the drying rate while preserving the physical, chemical, nutritional and organoleptic qualities of the final product [8]. In biological systems, oxidative stress results from an imbalance in free radicals and antioxidants [2]. Design of dryers for foodstuffs is a complex problem due to the peculiar characteristics of food systems[9].Grape is one of the world's largest fruit crops, with an

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approximate annual production of 58 million metric tons, and it is well known that the grape skins, seeds, and stems, waste products generated. To preserve food there are different food preservation methods that have been applied for many years, for example, fruit drying process. To produce raisins grape drying is done which is a very slow process[10]. The preservation of Grapes in the form of raisins is profit-making marketing in different countries where grapes are grown[11].

The total phenolic compounds are also closely related to grape ripeness and often used to evaluate grape quality [12]. Vegetables and fruits contain low level of fat high levels of vitamins, minerals, fiber and variety of substances that can be used as a health-promoting agents for humans, since they contain antioxidants such as vitamin c,tocopherol carotenoidsthey have the role for prevention of human diseases such as cancer immune depression[13]. Because of high nutritional value, raisin is the most important and popular dried fruits in the world [14,15]. In grapes about 40 terpenes have been identified; they can occur as hydrocarbons, alcohols, aldehydes, ketones, or esters[16]. Odorant molecules which are exhaled by natural products of vegetable origin are attracting more attention to the scientific sectors [17].

The flavored compound formed by Maillard reaction in the fruits and vegetables[18]. The quality characteristics of raisins are volatile compounds, and they are present in grapes as well as in raisins. In Raisin, the volatile compounds are present in both free-form and glycosidically bound-form[19]. Volatile flavor components of muscadine grapes have been investigated, however, those components responsible for characteristic muscadine grape aroma notes [20].

During any drying operation, one critical entity, competing with that of process economics, is quality preservation[21]. Sun natural drying is one of the most common traditional preservation methods used, in particular in non-industrialized countries[22]. From a nutritional perspective, one widely utilized quality criterion is used at the extent of ascorbic acid function during food processing. Selected as a test nutrient, due to is temperature- and moisture sensitivity, this parameter is indicative of the mildness of the drying process[23].

High-quality products are the present demand in the food market which requires dehydrated foods that could maintain at a very high level the nutritional and organoleptic properties of the initial fresh product[24]. Platelet aggregation and low-density lipoprotein (LDL) oxidation can be inhibited by the consumption of grape juice[25]. Design of dryers for foodstuffs is a complex problem due to the peculiar characteristics of food systems[26]

Polyphenolic compounds are gallic acid, catechin, anthocyanins, and resveratrol[1]. Polyphenols are one of the examples of plant secondary metabolites and these have several beneficial characteristics such as antioxidant, anti-aging, anti-carcinogenic, anti-inflammatory and it improves endothelial function[2].Polyphenols have gained much attention by food scientists, nutritionists, agricultural and food industries and consumers due to the association between the consumption of a diet rich in plant sources with disease risk reduction such as cancer and cardiovascular disease as well as type 2 diabetes .In various countries dried fruits are consumed regularly[3]. The shelf life of dried fruits has increased by using the process of removing moisture during its production[3]. Raisins contain phytochemical compounds such as flavonoids, alkaloids, terpenoids, and polyphenols. Alkaloids have antimicrobial activity and act as painkillers.

The main object of this study is to analyse the phytochemical compounds and to determine the quantity of gallic acid by high-performance liquid chromatography in three different types of raisins.

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2. MATERIALS AND METHODS

2.1 Plant material

Three types of dried raisins were purchased from a local shop of Kattigenahalli, Bangalore. Three types of raisin are a golden raisin, brown raisin, black raisin.

(A) (B) (C)

Figure 1.(A) golden raisin(B) brown raisin(C) black raisin

2.2 Chemicals reagents and instruments

All reagents, chemicals, and instruments which were used in this project belonged to the Biochemistry Department, Reva University.

2.3 Extraction of phytochemicals from raisin

Extraction of phytochemicals processed according to [2] with little modification, 10 grams of each sample was weighed and kept in a freezer at 20 degree centigrade for 14 days. The frozen samples were homogenized by using mortar and pestle. Homogenized samples were kept in a freezer at -20 degrees centigrade for the next 24 hours. Then each homogenized sample was dipped in 100 ml methanol separately for 24 hours in orbital shaker for better extraction at room temperature. Each extracted samples were filtered through Whatman filter paper in a separate conical flask. Then each filtrate was concentrated by using rotatory evaporator. The crude products were kept in dark place at room temperature.

2.4 Qualitative analysis of phytochemicals from raisins

Qualitative analysis of phytochemicals from the extracts of three different types of raisins was carried out according to the standard protocols.

2.4.1 Qualitative test for alkaloid: To 1 ml of raisin extract, 1ml of Mayer's reagent and few drops of iodine solution was added in three different test tubes. Formation of Yellow precipitated was as an indication of the presence of alkaloid.

2.4.2 Qualitative test for flavonoid: Raisins Extract products were heated with concentrated. Sulphuric acid in three different test tubes. In three test tubes. Formation of yellow color was as an indication of the presence of flavonoid.

2.4.3 Qualitative test for terpenoid: To 1 ml of raisins extract, 1 ml of conc.H2SO4 were added in three different test tubes and then heated for 2 minutes. Formation of greyish black color was as an indication of the presence of terpenoid.

2.4.4 Qualitative test for tannin: To 1 ml of raisins extract, 1 ml of Fecl3 was added in three different test tubes. Formation of green precipitate was taken as an indication of the presence of tannin.

2.4.5 Qualitative test for phenol: To 1 ml of raisins extract, 1 ml of Fecl3 was added in

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three different test tubes. Formation of greenish blue color was as an indication of the presence of phenol.

2.5 High performance liquid chromatography (HPLC) analysis

A quantitative test of gallic acid was performed by using High-Performance Liquid Chromatography (HPLC)containing Shimadzu Luna C-18 (250×4.6mm, 5µm) column. The concentration of the standard sample which is gallic acid was 20µg/ml. The concentration of the three samples were 10µg/ml each. The separation was achieved with acetonitrile containing 1% acetic acid in a linear gradient program, for a time interval of 20 minutes. Results were obtained by comparison of peak areas (λ_{max} = 254nm) from the three samples of raisin extract. (Sample 1 = 300mg extract, Sample 2 = 260mg extract, Sample 3 = 280mg extract) with that of standards 20µg/ml.

3. RESULTS

3.1 The results of phytochemical components qualitative screening from three different types of raisins were listed in the table.

Table 1: Qualitative analysis of phytochemicals

Qualitative analysis for phytochemicals had done from the extract of golden, black, brown raisins, the analysis result revealed that maximum alkaloid content in golden raisin than black raisin and followed by brown raisin; Maximum phenolic content in golden and brown raisin than black raisin; maximum terpenoid content golden raisin than brown and black raisin. Terpenoid and flavonoid content is maximum in golden and brown raisin than brown and black raisin.

Phytochemicals	Golden Raisin	Brown Raisin	Black Raisin
Alkaloid	+ + + +	+ +	+ + +
Phenol	+ + +	+ + +	+ +
Tannin	-	-	-
Terpenoid	++++	++	+++
Flavonoid	+++	++++	+++

3.3 HPLC analysis

HPLC quantitative analysis for raisins extract has done and the result revealed that 15.15 mg of Gallic acid was quantified in 300mg from golden raisin

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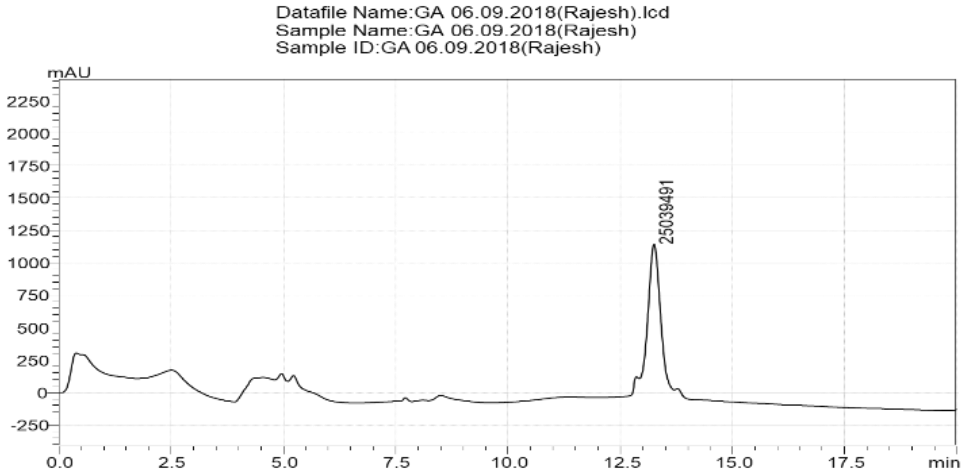
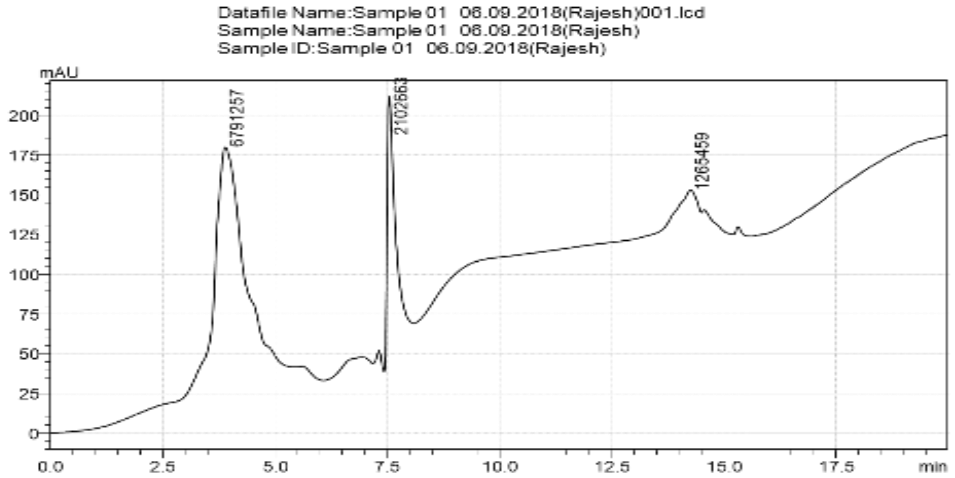
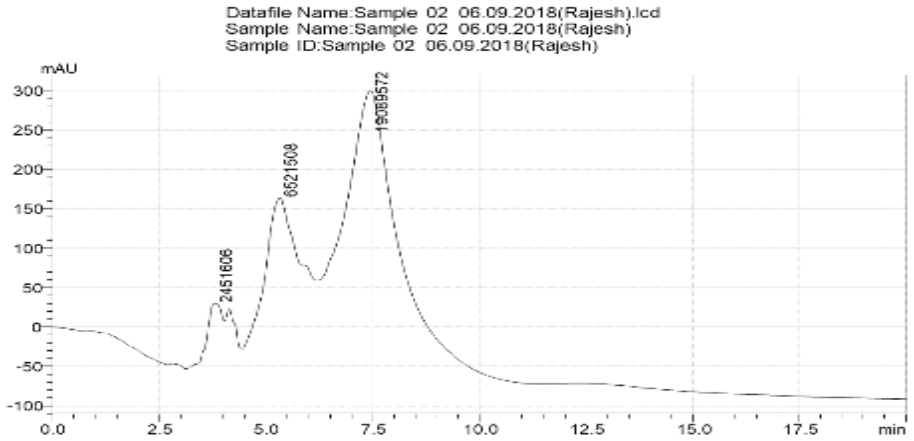


3.3.1 Figure: Chromatogram of standard (Gallic acid)



3.3.2 Figure: Chromatogram of golden raisin extraction



3.3.3 Figure: chromatogram of brown raisin extraction

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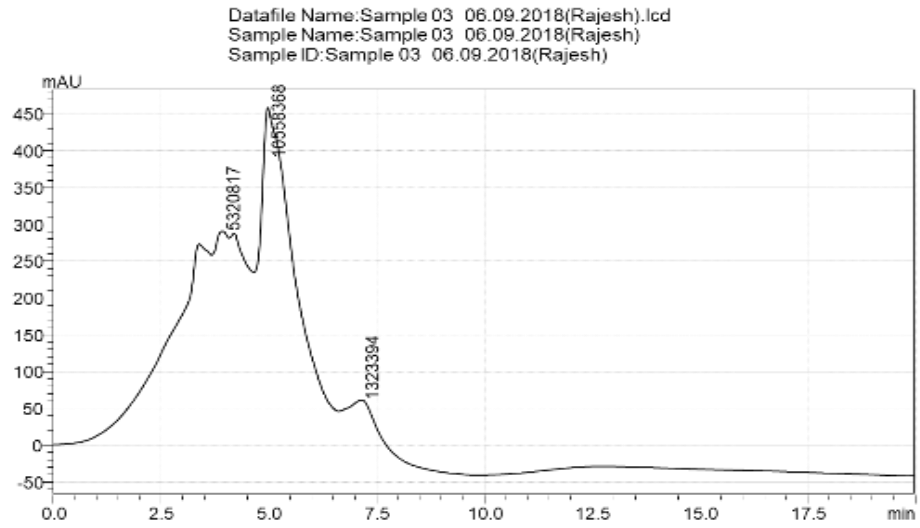

3.3.4 Figure: Chromatogram of black raisin

4. DISCUSSION

In the previous study, investigators suggested that four raisin *(Vitis vinifera L.)* varieties, Chriha, Razeki, Assli, and Meski, were evaluated for total phenolic content (TPC), total o-diphenol content (ToPC), total flavonoid content (TFC), total condensed tannin (TCT), total carotenoid content (TCC), and total anthocyanin content (TAC). Individual phenolic profiles were determined by HPLC. The results revealed that the four raisin varieties had considerable phenolic content [2]. In the present study, qualitative analysis for phytochemical compounds was done by using the standard method for the phytochemical test from fruits extract which revealed that each raisin contained alkaloid, flavonoid, terpenoid, phenol. Gallic acid was quantified by HPLC analysis which revealed that golden raisins contained higher amount (15.15mg/300mg of golden raisin) of gallic acid compared to brown and black raisins.Due to the presence of phytochemical compounds and gallic acid, our investigators suggests that the raisins may have anti-inflammatory, antioxidant, anti-allergen protection and anticancerous property.

5. CONCLUSION

In this study methanolic extract of raisins were analysed for phytochemical compounds and amount of gallic acid and it's in the extract.The results of this study confirm the presence of phytochemical compounds in all three types of raisins and gallic acid is mainly present in the golden raisin.

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HPLC ANALYSIS OF BLUEBERRY FRUIT *VACCINIUM CORYMBOSUM* EXTRACT FOR GALLIC ACID ESTIMATION

V.Veeraraghavan[1*],N.R.Neeraja[1]
[1]School of Biochemistry, REVA University, Bangalore-560064, India

ABSTRACT: Phenolic acid falls under the category of phytochemical called polyphenol. They are found in the variety of plant based foods especially in fruits and vegetables in higher concentrations. Blueberries (*Vaccinium* sps.) are rich in polyphenols and others secondary metabolites. Polyphenols are used in treatment of cardiovascular diseases, neurodegenerative diseases and especially in fighting against cancer. In regards of polyphenols, here we analysed the gallic acid in blueberry fruit.Blueberries fruits were homogenized in methanol andcollected the methnaolic extract and futher subjected for the HPLC analysis. Gallic acid (3,4,5 trihydroxybenzoic acid) is a secondary metabolite present in most plants. This metabolite is known to exhibit a range of bioactivities including antioxidant, antimicrobial, anti-inflammatory, and anticancer. *Vaccinium corymbosum* were extracted by methanol and evaluated. Qualitative analysis were carried out; Thin layer chromatography for the presence of phenolic compounds and High performance liquid chromatography for the quantification of the compounds. Spectroscopic analysis were performed to detect the properties or the concentration of the sample.

Keyword: Polyphenols, Blueberries, Gallic acid.

[*]**Corresponding author:** Dr.V. Veeraraghavan
School of Biochemistry, REVA University, Bangalore-560064, India.
Email: niraja1245@gmail.com

1. INTRODUCTION

Blueberries have positive effects on human body in respect of antioxidants, anti-inflammatory, protective effects on neuronal stress, diabetes intervention, benefit to cardiovascular health, improvement in cognitive performance, modulation of intestinal microbiota and improvement of renal function[1].The most important characteristic of blueberries are their bioactive properties and one of the richest sources of bioactive substances among fruits and vegetables and antioxidant phytonutrients, thus making them effective in inhibiting oxidation of human low-density lipoproteins and preventing or alleviating various human diseases caused by oxidative stress;[2] while composition and content of phenolic compounds in blueberries have changed in relation to variety, period, as well as to locality of growing.

Polyphenols and phenol compounds represent one of the most numerous and most represented groups of plant metabolites and form part of the human diet. They are products of secondary metabolism of plants possessing one or more aromatic rings bearing one or more hydroxyl groups with over 8,000 structural variants and generally are categorized as phenolic acids and analogs, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others based on the number of phenolic rings and of the structural elements that link these rings[3].Plant polyphenols are substances widespread in almost all plants, particularly in the leaves, flowers, seeds, fruits. One group of polyphenols includes a wide and diverse range of compounds from simple phenolic acids to high polymerized tannins. More than 8.000 phenolic compounds are known, only a few hundred are identified in the

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edible parts of plants. Very bright coloured fruit is an important source of polyphenolic substances. Polyphenolic substances contained in foods of plant origin are intensively monitored plant components at present[4]. Their impact on human health is discussed for professional and general level, the views of t heir operation are not fully uniform.The content of phenolic compounds in natural materials is quite variable, depending on each type of crop,also their varieties. It is genetically conditioned and affected by agronomic and environmental soil and weather conditions. The different categories of polyphenols present in blueberries are responsible for that beneficial effects. These polyphenols include anthocyanins, flavonols, catechin and proanthocyanins as well as hydroxycinnamic acid.

One of the major phenolic acids, Gallic acid (or gallate) is a benzoic acid of great importance for the formation of a so-called Galatotanin-hydrolyzable tannins group formed by a unit of sugar and a variable number of phenol acid molecules.Gallic acid falls under this category and appeared to be present in their ester forms, probably as glucoside esters. Gallic acid is a crystalline solid, slightly colourless or slightly yellow. Its molecular weight is 170.11954 g/mole and its molecular formula is C7H6O5. The aim was to analyse Gallic acid in blueberry fruit extract[5].

2. MATERIAL AND METHODS

2.1 Plant Sample

Fresh blueberries fruit was collected from the local market (KR Market, Bangalore, India) for the experiment and remaining sample kept in deep freezer for the further use. *Vaccinium corymbosum*, the northern high bush blueberry (the whole fruit).

2.2 Extraction Procedure

The edible mature fruit of *Vaccinium corymbosum*, the northern highbush blueberry, is a North American species of blueberry. It is native to eastern Canada and southern United states. This is the most commercially grown blueberry in present-day North America[6]. After the collection of blueberries, it is stored in a deep freezer of about 8 degree celcius for several days (approximately 3-4 weeks) i.e freeze drying process[7]. Fried dried process of blueberries overall yield of extraction is high. A soxhlet apparatus was used for the extraction purpose (Fig.1). The solvent used was methanol. Methanol was used because of its ability to extract a wide range of compound polarities, ranging from polar to water soluble also generally found to be more efficient in extraction of lower molecular weight polyphenols[8].The boiling point of methanol is 64.7 degree celcius[9]. For the extraction purpose, about 15.76 grams (approxiamately) was loaded into the thimble and 800ml of the methanol was added into the flask refluxed for 4 ½ hours. Three soxhlet has been run for about 6-7 cycles each soxhlet. The extract obtained were then subjected to the rotary vaccum evaporator for the removal of solvent, methanol. The rpm set was 70rpm and the temperature was initially set at 50 degree celcius. Finally 4ml (approximately) of ethanol was used for the removal of sample from the round bottom flask after subjected to rotary vaccum evaporator[10].

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Figure 1. Soxhlets method of extraction procedure and concentrated fruit extract in petri plate.

2.3 Thin Layer Chromatography:

The extract is subjected for condensation using rotary vaccum evaporator and the concentration of extracts were made to 1mg/ml.The percolated TLC plates were made up of silica gel as an absorbant was used. The extracts of blueberry were spotted on TLC plates and placed in a solvent system containing a mixture of chloroform: ethyl acetate: formic acid in the ratio 5:4:1[11]. Ferric chloride is used as a spraying reagent.Then it is subjected to UV chamber[12]. The presence of blue colour on TLC plate shows the presence of phenolic compound in the *Vaccinium corymbosum* (whole) extract[13]. (Fig. 2).

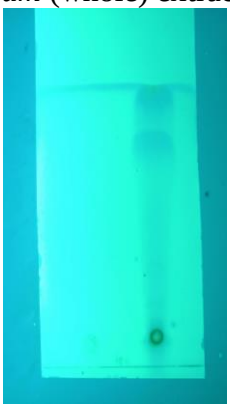

Figure 2. TLC image of fruit extract.

2.4 Determination Of Total Phenolic Content:

Phenolics concentration in plant extracts was determined using spectrophotometric method. UV/VIS spectrophotometer was used for absorbance measurement in the visible regions.The extracted methanolic extract of while blueberry fruit was used as sample.

Reagents And Chemicals Used:

Folin-Ciocalteau reagent, methanol, Gallic acid and sodium carbonate. Milli-Q water was used[14]. The freeze dried blueberry sample of 20mg was grinded using methanol.Centrifuged for 5 minutes at 10000rpm. The supernatant of about 300 microliter is then collected.Add 600 microliter of folin- ciocalteau reagent is added i.e 10% FC reagent.Add 2400 microliter of 700mm of sodium carbonate is added.Keep at room temperature for about 2 hours.Gallic acid was used as standard. Initially 6.8mg of gallic acid was added in 40ml of water. 0.0035 grams of gallic acid is used as standard. Blank solution

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was prepared by taking 300 microlitres of water in a falcon tube. To that 600 microlitres of folin – ciocalteau reagent and 2400 microlitres of 700mm sodium carbonate was added. Keep it at room temperature for two hours. The absorbance was read at 765nm and the calibration curve was calculated[15].

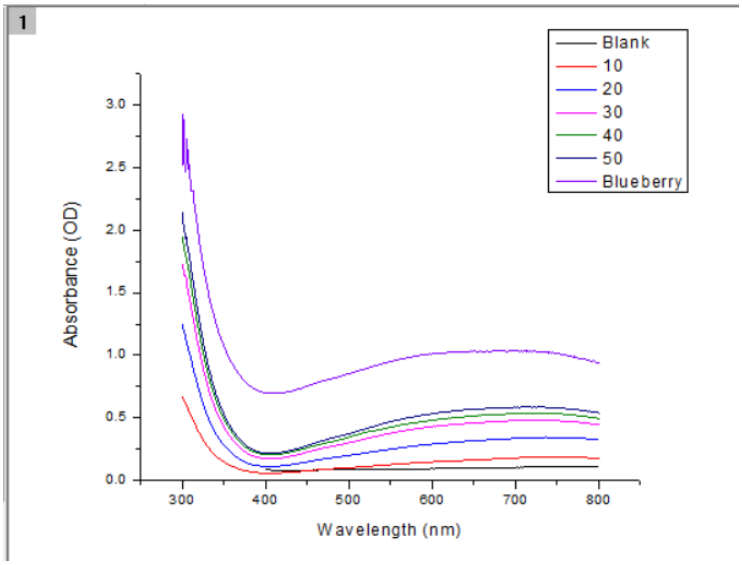

Figure 3. Total phenolic analysis of *Vaccinium corymbosm* by UV-Visible spectroscopy.

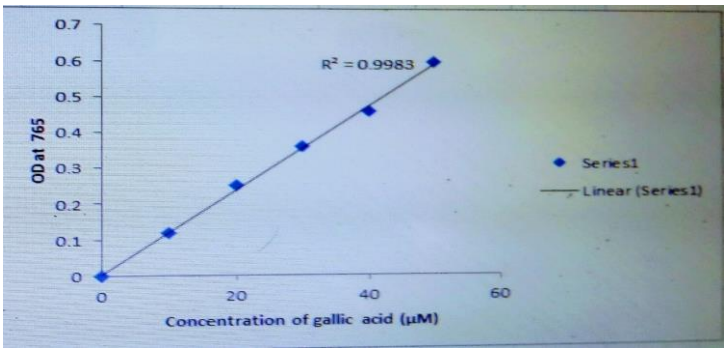

Figure. 4

2.5 HPLC Analysis

Blank Sample Preparation:

Methanol was used as blank because methanol was used as a solvent for extraction purposes.

Standard Preparation:

Gallic acid was used as standard. The preparation was 1mg of gallicacid in 1ml of methanol.

Sample Preparation:

Two different concentration of samples were prepared. Sample1 is concentration of 1mg of extracted whole blueberry in 1ml of methanol. Sample 2 is concentration of 2mg of extracted whole blueberry in 1 ml of methanol[16].

Solvent Preparation:

The solvent preparation was 300ml. 210ml of distilled water and 90ml of acetonitrile and

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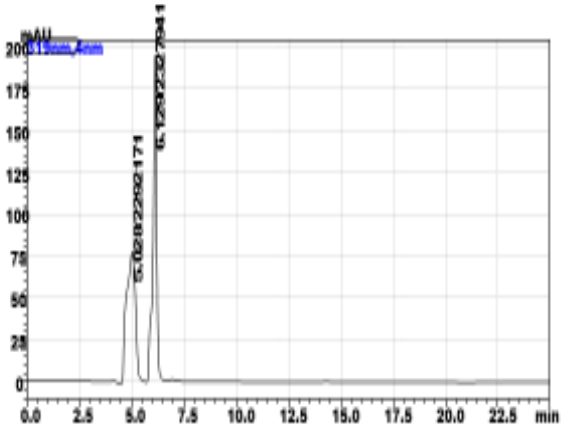
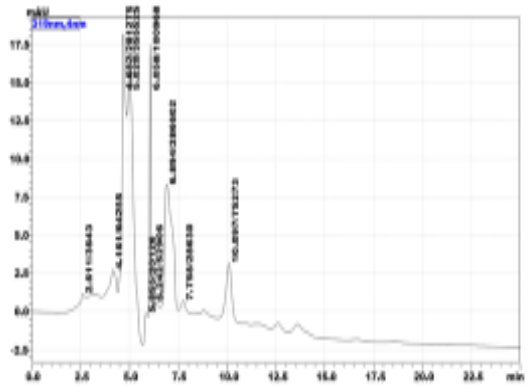
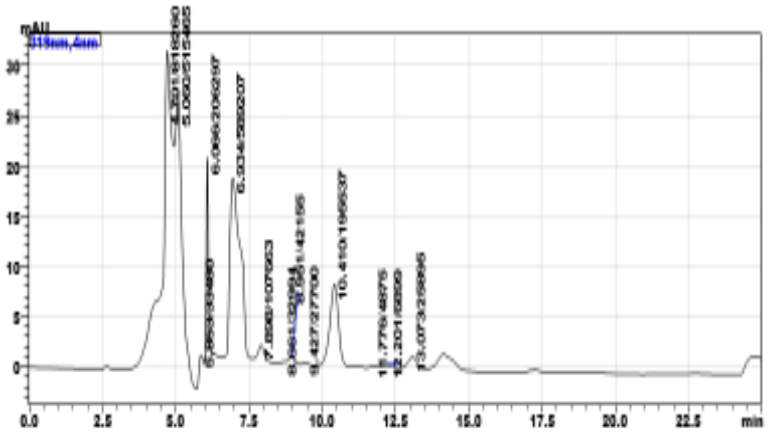
acetic acid mixture.Run time was 25minutes.The volume injected in HPLC is 20 microlitres.

a) Standard Gallic acid- 1mg/ml of methanol.



b) Sample 1- concentration of 1mg/ml of methanol





c) Sample 2- concentration of 2mg/ml methanol

3. RESULTS AND DISCUSSION

Plants have been used since ages because of the various biological properties due to secondary metabolites that are synthesised in it. This investigation reveals the phenolic acids presence in *Vaccinium corymbosum.* These bio active phytochemicals are the basis in the treatment of several diseases and also these compounds generate characteristics physiological action on humans. The presence of phenolic compounds are confirmed by TLC, As it gave a blue colour when viewed under UV chamber[17]. Methanol was found effective in extracting secondary metabolites. HPLC analysis was done to find the gallic acid content in the sample by various concentrations. The peak from the analysis proves that the presence of gallic acid in it.

4. CONCLUSION

Seeing the results of all the analysis it can be concluded that *Vaccinium corymbosum* produces secondary metabolites like phenolic compounds of medicinal value. The biological activity of gallic acid if of great importance[18].TLC confirmed the presence of phenolic compounds and HPLC confirmed the targeted gallic acid presence by the peaks. Methanol

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was found the best suited sample for extraction purposes. The approaches described in this work can be useful for the evaluation of gallic acid from various sources of all parts of the plant and upto the final product in the development of drugs.

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TISSUE SPECIFIC VARIATION OF STIGMASTEROL CONTENT IN *CALOTROPIS GIGANTEA*

V Veeraraghavan[1*], B Pooja[1], Hemavathi B S[1], Ramesh Kumar Kushwaha[1]
[1]Department of Biochemistry, REVA University, Kattigenahalli, Bengaluru

ABSTRACT: Phytosterol and stanols, naturally occurring phyto-compounds, resembles structurally to cholesterol shows outstanding results in their cholesterol lowering activity. Plant sterol and/or stanol significantly reduce plasma total and LDL cholesterol. The main purpose of the plant, *Calotropis gigantea*, was to determine the anticholesterol and antilipidemic activity in the different tissue extracts of the aerial parts (tender stem, whole flower, leaves, fruit and latex). Extracts were prepared using methanol as organic solvent by Soxhlet and maceration methods. Phytochemical screening tests have confirmed the presence of sterols and stanols in the 5 different extracts during qualitative studies. Thin layered chromatography was applied for the detection and quantifying the specific phytosterol (stigmasterol) comparing the standard. Reverse phase- HPLC and mass spectrophotometry needed to be performed for the analysis of the activity and the structural function for each bioactive compound of the extracts. Hence, the investigation of the extracts for stigmasterol, as reported and analyzed, can be used as biopotential therapeutic agent.

KEYWORDS: *Calotropis gigantea*, phytosterol, anticholesterol activity, phytochemical screening, TLC.

[*]**Corresponding author:** Dr.V. Veeraraghavan
School of Biochemistry, REVA University, Bangalore-560064, India.
Email: veeraraghavan@reva.edu.in; Mobile: 9880241577

1. INTRODUCTION

Family Asclepiadaceae, commonly known for its latex production and widely opted for its medicinal value[3]. *Calotropis gigantea*, among all the species, which is recognized for its traditional medicinal properties, because of the variety of bioactive compounds been isolated from different parts of the plants and are analyzed pharmacologically. It is significantly reported for the analgesic activity, antimicrobial activity, antioxidant activity, anti-pyretic activity, insecticidal activity, cytotoxic activity, hepatoprotective activity, pregnancy interceptive properties, purgative properties, procoagulant activity and wound healing activity[4]. This study is mainly based on the anticholesterol and antilipidemic activity and analysis of particular bioactive compound (phytosterol) in different tissue extracts of *Calotropis gigantea*.

Table 1. Taxonomical classification of *Calotropis gigantea*

Kingdom	Planatae
Subkingdom	Tracheobionta
Family	Apocynaceae
Subfamily	Asclepidiaceae
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Dicotyledones

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Sub class	Asteridae
Series	Bicarpellatae
Order	Gentianales
Genus	*Calotropis*
Species	*Calotropis gigantea*

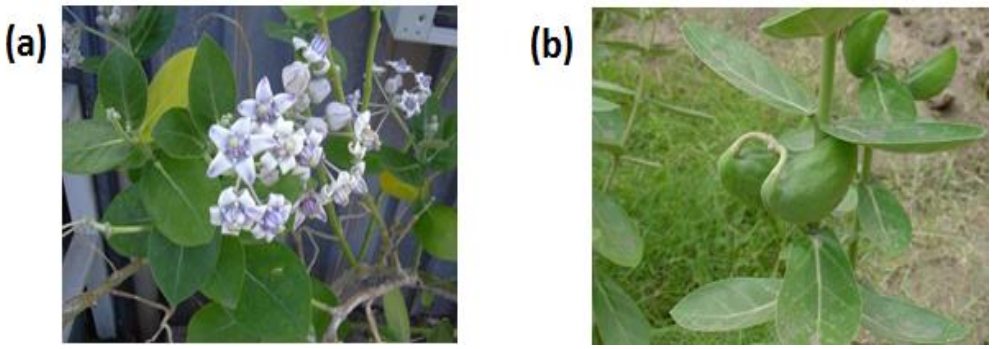

Figure 1. (a)Flower (b) Fruit of *Calotropis gigantean*

Steroid compounds, phytosterol, are naturally occurring plant sterols and stanols, similar to cholesterol, varying structurally only in carbon side chains and/or presence or absence of double bonds. They occur naturally in the plants either esterified with fatty acids in the cell membrane or in free form within the cell. These are predominantly known for their properties of lowering blood cholesterol level. Plant sterols and stanols are mainly different in their structural basis by the absence or presence of double bonds (Figure 2). Sterols have a double bond in their sterol ring, hence forming unsaturated structures; whereas stanols lack double bond in their structure forming saturated compounds[2].

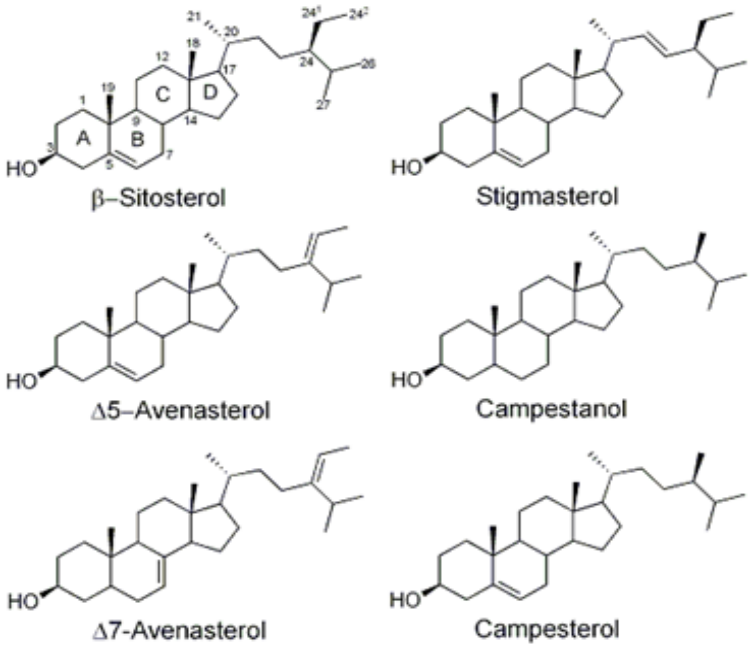

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Figure 2. Structural difference between phytosterol and stanols

The elevated cholesterol level in blood was treated by the phytosterol for the first time in 1952, demonstrating its pharmaceutical importance. In human intestinal lumen, the intake of the plant sterols and stanols have shown its ability to reduce the formation of micelles with cholesterol and inhibiting cholesterol absorption by 30-40%, hence lowering serum total and LDL cholesterol concentration. Saturated stanols are found to be more effective than the unsaturated forms in lowering cholesterol levels[1].

Emulsified phytosterols form micelles due to the action of secreted bile salts in the small intestine, like cholesterol and lipid molecules. Cholesterol esterase and pancreatic lipase enzyme hydrolyze esterified sterols and stanols into free phytosterols. These are absorbed into enterocytes by transporters which are also involved into cholesterol absorption. There the phytosterols combine with cholesterol, triglycerides and apolipoproteins to form chylomicrons, which are secreted into the lymph and then transported to the liver via bloodstreams. They are either used up for the formation of bile salts or incorporated into very low density lipoprotein, and then secreted into the bloodstreams. These VLDL are converted into low density lipoprotein for cellular uptake. Phytosterol have been found at high concentration in the tissues of lungs, adrenal cortex, intestinal epithelia and ovaries. The transport of phytosterol from the lumen into intestinal enterocytes is lower than that of the cholesterol and is dependent on the structure of the different sterols and stanols[6]. Taking into consideration, the aim of the study was to conduct qualitative and quantitative analysis for the tissue specificity of stigmasterol in the plant. In context of phytosterol importance here we analyzed the tissue specific presence of stigmasterol.

2. MATERIALS ANDMETHODS

2.1 Plant material collection and preparation of the extract

The plant sample was collected from the surrounding village of REVA campus, Sathnur, Bangaluru. The aerial parts of the plant were collected, which included tender stem, young leaves, whole flower, mature fruit and the latex. Stem, leaves, flower and fruit were washed and dried properly under the shade. The latex was directly collected into the tube and stored in freezer at 20ºc for the desiccated and extraction process. The stem were cut into small pieces and dried under in shade at room temperature for 4 to 5 days. The leaves were torn into pieces and were dehydrated in room temperature. The fruit skin and flower were also dried under shade for 5 to 6 days. The dried samples were grinded into fine coarse powder and were sieved to collect the dried sample[14].

20ml of latex was collected and dehydrated at 100ºc in water bath for 3 hours, the semisolid latex material was scraped out and used for the soxhlet method. Methanol was the solvent and the extraction method was carried out at 60ºc for 6 repeated cycles. The extract was collected in the round bottle flask and proceeded with the rotary vaporator. The crude sample was collected and desiccated for future use.

250 grams of tender stems and leaves were dried and powdered for the soxhlet procedure of extraction at 55ºc till the solvent turned colorless using methanol as organic solvent. The extract was evaporated in rotary vaporator till the liquid extract turned semisolid or solid.

The whole flower and the fruit were allowed to dry and proceeded with the soxhlet method at 55ºc and repeated cycles till the methanol becomes colorless in tumble. Evaporated in rotary vaporator and crude sample was collected.

The crude sample of all the five aerial (Figure 3) extracts were analyzed for the confirmation

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of phytosterols and the quanlification of stigmasterol was carried out in REFERENCES to the standard[15,16].

2.2 Phytochemical analysis

The preliminary phytochemical screening of extracts of *Calotropis gigantean* was carried out according to the standard methods. The presence of overall phytosterol and triterpenoids was evaluated. Standard protocols were referred for the confirmation of the phytochemicals such as sterols and terpenoids with Libermann Burchard`s analysis and Salkowski's test[11].

Salkowski's Test for detection of terpenoids

Extract were treated with 2ml chloroform and then filtered. The filtrate was treated with 1ml concentrated sulphuric acid and was allowed to stand. Appearance of golden yellow color or reddish brown indicates the presence of triterpinoids.

Libermann Burchard's Test for detection of sterols

Extract were treated with 10ml chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Equal amount of concentrated sulphuric acid was added. Formation of brown ring at the junction confirms the presence of phytosterols.

2.3 Thin Layered Chromatography for various crude extracts

Various tissue extracts of Calotropis were obtained by subsequent and sequential Soxhlet methods with methanol as solvent. 150ml of methanol was used for the soxhlet extraction.

The crude sample were diluted to the concentration of 1mg/ml with methanol.

The stigmasterol stamdard was used for the TLC analysis. 1mg of standard stigmasterol compound was dissolved in 1ml of methanol, forming concentration of 1mg/ml.

All the 5 different crude extract was applied separately on silica gel 'G' coated and activated thin (0.2-0.3 mm) glass plates along with the standard REFERENCES samples of stigmasterol. The plates were developed in an organic solvent mixture of Benzene and Ethyl acetate (85:15 v/v), air dried, sprayed with 50% sulphuric acid and subsequently heated at 100 ºC for 10 minutes.

After the application of the sample on the plate the plates were kept in TLC glass chamber (solvent saturated) than mobile phase was allowed to move through adsorbent phase up to 3/4th of the plate. TLC was performed for all the samples referring with the standard[7].

3. RESULTS

3.1 Phytochemical analysis

Preliminary biochemical screening of various tissue extracts of *C. gigantean* suggested the presence of various phytosterols which have medicinal properties. As shown in table 2, all the extracts showed the positive result for the terpenoids and sterols. The Flower extract and latex have the high concentration of the sterols, whereas the fruit may not have the steroid compounds. Stem seemed to be rich in the terpenoids and sterols as well.

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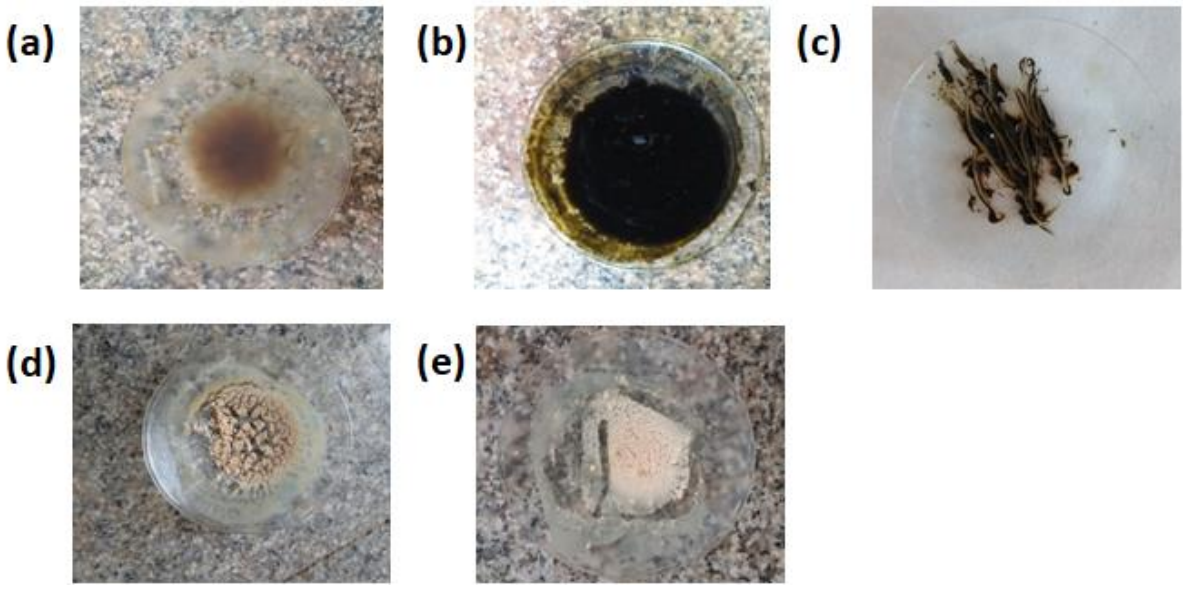

Figure 3. Crude extracted samples of (a) stem, (b) leaf, (c) fruit, (d) latex and (e) flower

Table 1: Phytochemical analysis of *Calotropis gigantea* extracts

Phytochemical test for different crude extracts	**Salkowski's test for terpenoids**	**Liebermann Burchurd's test for sterols**
Stem	++	+
Leaves	+	+
Fruit	+	-
Flower	-	++
Latex	+	++

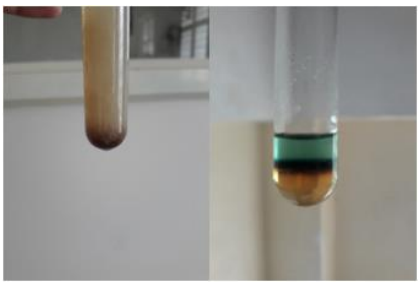

Figure 4. Phytochemical Screening Tests

3.2 TLC analysis

The spots coinciding with the authentic sample of stigmasterol was marked on TLC (Figure 5a) and 5 spots of all the samples were coinciding with the standard sample of stigmasterol.The Rf value of the standard being 0.58 and the other spots of stem(0.52), fruit(0.48), flower(0.50) and latex(0.50). The observation showed negligible spot or mark for the leaf extract.

In Figure 5b, graphical representation shows the overall variations in concentration of sitgmasterol in different tissue. The standard spot(1mg/ml) and all the plant extracts, namely,

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stem, leaf, latex, fruit and flower, which were extracted in the methanol and dissolved in 1ml of methanol, showed low to high concentration variations. The calculation revealed the deviation of the stigmasterol in the different tissue from the 10µl of standard stigmasterol.
The maximum concentration of stigmasterol was revealed in flower (7.64µg/10µg) and latex (7.59µg/10µg) followed by stem (7.5µg/10µg) and fruit (7.49µg/10µg) with minimal concentration in leaf (6.6µg/10µg), comparatively.

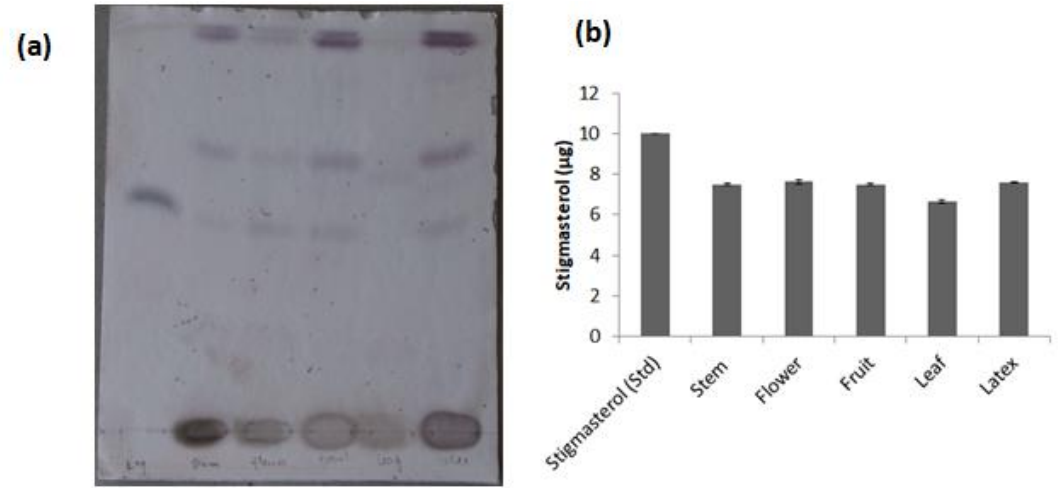

Figure 5. (a) Thin Layer Chromatography (b) The quantitative measurement of stigmasterol present in different tissues.

4. DISCUSSION

In the investigation, it is confirmed for the presence and absence of the steroidal compounds in the plant extract. The phytochemical analysis showed the presence of terpenoids and sterols in different extracts with the variation in the concentration of the compounds. Thin layer chromatography is usually done for a better identification of the bioactive compounds. The TLC result clearly demarcated the concentration and the quality of the stigmasterol in the different extracts, as the Rf values of each extract coincides with the standard sample, except the leaf. This indicated the negligible amount of the stigmasterol rather than showing absence. The plant, *Calotropis gigantea,* can be used as a better remedy for the cholesterol related issues as well[8,911,14,16].

5. CONCLUSION

Current investigation reports on preliminary screening of phyto-chemicals of the *Calotropis gigantea* extracts. The results concluded the efficacy methanolic extract of different parts of plants have the bioactive compound required for the research. Which confirms the presence of sterols and stanols in the extracts can be used as the bioactive therapeutical compound against the cholesterol related health issues.

CONFLICT OF INTEREST

None

ACKNOWLEDGEMENTS

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SECONDARY METABOLITES IN *PHYLLANTHUS EMBLICA* FRUIT AND THEIR ANTIOXIDANT PROPERTY

Jayasri P[1], Jayalakshmi Sinde[1], Kavya C M[1]

School of Biochemistry, REVA University, Kattigenahalli, Bangalore.

ABSTRACT: The present study was designed for the antioxidant propertiesof *phyllanthus emblica* or Indian gooseberry fruit which belongs to the family of Euphorbiaceae. The fruits of Amala are extensively used in the Ayurvedic medicine prepartion and are suppose to increase defence against disease. It has a beneficial role in many diseases like cancer, diabetes,liver diseases, ulcer, anaemia, heart problems and also is an important constituent in hepatoprotective formulas. The fresh or dried whole fruit of *phyllanthus emblica* is used in Ayurveda(Indian),Chinese,Egyptian customary system of medicine as a powerful rejuvenator, throughout India as a medicinal food with antiinflammatory and antipyretic effects. The fruit contains a series of diterpenes like gibberrllins, triterpene lupeol, flavonoids and polyphenols with pharmaceutical properties and antioxidant activities. Qualitative phytochemical analysis of our extract revealed the presence of major phytocompounds like alkaloids, flavonoids, phenols, saponins and Tannins. HPLC analysis revealed the presence of 64µg of gallic acid in 1mg of ethanolic extract of amla fruit. The total phenolic contentof the ethanolic extract of *Phyllanthus emblica* fruits were found to be1.14mg GAE/g. The hydrogen peroxide scavenging assay showed good scavenging activity of the ethanolic amla fruit extract. The IC_{50} value and the scavenging percentage of ethanolic extract of amla fruits were found to be 0.066µg/ml and 50.0 respectively. The IC_{50} value and the scavenging activity of standard ascorbic acid were found to be 0.24µg/ml and 50.0 respectively. This fruit extract secondary metabolites can be considerd for anticancer property in future studies.

KEYWORDS: *Phyllanthus emblica,* HPLC analysis, Phytochemical analysis,total phenolic content, hydrogen peroxide scavenging assay, antioxidant property, anticancer property.

*Corresponding authors: Jayasri.P
Email: jayasri.p@reva.edu.in
Mob No. 9902402820

1. INTRODUCTION

Phyllanthus emblica, medium sized deciduous tree, which belongs to the family euphorbiaceae, it is mainly distributed in most of the tropical and subtropical countries. 550 to 750 species and 10-11 subgenera are present[1-2]are present in the phyllanthus genus .*Phyllanthus emblica* is considered as “the best among sour fruits”, and it is also considered as “the best among the rejuvenator” according to the Charaka Samhita and Sushruta Samhita[3].In Ayurvedic and Unani system of medicine, one of the most commonly used herbal drug is the*Phyllanthus emblica* fruit[4].In the preparation of the important medicines like Triphala and Chyawanprash, Phyllanthus emblica is the significant ingredient[5].

Phyllanthus species contains high amount of vitamin C content[6].These species are also reported to be antipyretic and analgesic[7]. *Phyllanthus emblica* is also known as Amla or Indian gooseberry[8]. Amla contains numerous chemical constituents like tannins,alkaloids,phenols which shows biological activity[9].It is also one of the important sources of amno acids and minerals[10].Recent research studies shows that Amla is reported to be antitussive[11],anti-atherogenic[12],and adaptogenic[13]. The amla fruits when compared to the whole plant contains 28 percent of total tannin content.Emblicanin A and

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Emblicanin B are the two hydrolysable tannins present in the amla fruits[14].

Amla can be used as a energy refiller and urinary trouble frustration(diuretic) agent[15]. Amla can also be used as a febrifuge for the treatment of fever[16] and also as immune system enhancer[17]. It is also used in anaemia therapy[18]. It also helps to get relief from headache[19] and constipation problems[20]. It is also good for digestive system[21] and prevents skin damage[22]. It also promotes hair growth and good for reproductive health[23].Amla also shows Antivenom activity[24].

Oxygen is an important element to maintain homeostasis in cells for aerobic organisms.The processes which are involved in the production of the energy demands oxygen,which undergoes in the formation of free radicals and also called as Reactive oxygen species(ROS). These species such as hydrogen peroxide and superoxide anion are produced as byproducts of many biological reactions. These ROS causes oxidative damage in every cellby reacting with the molecules present in the cell. Oxidative stress is an important factor which is involved in the pathogenesis of many diseases.The ROS are important agents for the pathogenesis of age related disorders such as asthma,inflammatory arthropathies,parkinson's and alzheimer's diseases and also responsible for human aging[25].

Antioxidants are substances that can prevent or slow down damage to cells caused by free radicals and unstable molecules.These free radicals are capable of continously reacting with other molecules leading to chain reactions.Antioxidants such as thiols or ascorbic acid are capable of terminating these chain reactions. Hence these antioxidants are also called as free radical scavengers. Herbal plants possess good antioxidant property.

Many research studies have shown that natural phenols and flavonoids plays an important role in inhibiting or treating the cellular damage which is caused by the oxidative damage.The present investigation was aimed to assess the antioxidant activity of *Phyllanthus emblica* fruit extract to advocate the importance to use in medicine and also to use as a dietary supplements.

2. MATERIALS AND METHODS

2.1 Plants material collection and preparation of the extract

The mature fruits of *phyllanthus emblica* fetched from the local market Yelahanka, Bangalore, Karnataka. The fruits were cleaned by washing under running tap water and these fruits were cut into small pieces.Then these fruits were shade dried with occasional shifting for two weeks and dried fruits were powdered by using a mechanical grinder.The dried powder passed through sieve and stored in a tight container.25 grams of air dried powder of amla fruits were refluxed with 150ml of ethanol of 45^{o}C for threecycleswith the help of Soxhlet condensor and the mixtures were filtered. With the help of vacuum rotary evaporator,the filtrates were evaporated. The stock solution of ethanolic fruit extract was prepared by diluting with different solvents to obtain a final concentration of ethanolic extract [26].

2.2 Phytochemical Analysis of *Phyllanthus emblica* fruit extract

The preliminary Phytochemical screening of extracts of *Phyllanthus embilica* was carried out and the important phytochemicals like phenols, tannins, flavonoids, saponins was evaluated according to the standard protocols[27]. The presenceof phytochemicals such as tannins and phenolic compounds with ferric chloride test was confirmed. Flavonoids with alkaline reagent test and saponins with the ability to produce stable foam.

2.3 HPLC Analysis

Extracted samples were analyzed by high performance liquid chromatography [HPLC] containing [Shimadzu]C-18(250×4.6mm,5µm)column. 20mM monobasic phosphate buffer

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was used as amobile phase A which was prepared separatly and gradient methanol was used as mobile phase B.The mobile phase is eluted as per subsequent gradient programming. The pH of the mobile phase A is upheld at 4.5 using 10% phosphoric acid. Water and methanol was used in the ratio 3:2 as diluent during the course of the experiment. The detection wavelength was fixed at 272nm. The elution was completed at a flow rate of 1.0ml/min.A standard stock solution of gallic acid was prepared using the diluent. Serial dilutions were made using the diluent to formulate the different concentrations of gallic acid. By scheming peak area against sample concentration for each ingredient, calibration curve was done.1mgof finely powdered extract of *phyllanthus emblica* fruit was weighed accurately on the electronic balance and dissolved in 1ml diluent. 20 micro litre of this solution was injected.The peak areas attained were related to the slopes and intercepts from the calibration data to determine the concentration of the gallic acid in the ethanolic extract [28].

Gradient Programming of HPLC

Time(min)	Mobile Phase A	Mobile Phase B
0	95	5
8	90	10
12	95	5

2.4 Determination of Total PhenolicContent of *Phyllanthus emblica* fruit extract

Total Phenol contentwas determined by Folin-Ciocalteu reagent method descibed by Slinkard and Singleton with slight modifications [29]. A dilute extract of amla fruit (1.0µg/ml) was prepared.The standard stock solution of gallic acid was prepared (0.025mg/100ml) using distilled water and various concentrations of 0.25,0.50,0.75,1.0,1.25µg/ml were obtained by serial dilutions with distilled water.5 ml of FC reagent(1:10 dilution) was mixed with the 1ml of each sample and incubated for three minutes. 4ml of Na_2Co_3 was added to the mixture.The mixtures were incubated for 15minutes at 45^{o}C. With the help of UV spectrophotometer, Absorbance was measured at 765nm using water with FC as blank. The assay was accompanied concerninng the standard gallic acid concentration. Results were expressed in terms of gallic acid equivalence (GAE) in micrograms.

2.5 Hydrogen Peroxide Scavenging Assay

Among reactive oxygen species, the least reactive molecule is Hydrogen Peroxide. Under physiological pH and temprtaure it is stable in the absence of metal ions. Hydrogen peroxide can be produced from superoxide anion by superoxide dismutase by a dismutation reaction. In the presence of metal ions, it can produce the radical hydroxyl ion. Using phosphate buffer (pH 7.4), a solution of 40mM H_2O_2 was prepared. Ascorbic acid was used as the standard and solution of different concentratins was prepared. The solution of ethanolic extract of *phullanthus* fruit of 0.01mg/ml was prepared using distilled water and also solution of different concentrations was prepared. The solution was added to the hydrogen peroxide(88.63µl)solution. Phosphate buffer without hydrogen peroxide was used as a blank. The absorbance of hydrogen peroxide at 560 nm was determined and Scavenging activity of the extract and the standard compound was calculated using the given formula[30].

$$\% \text{ Scavenging activity}(\%) = \frac{(\text{OD of control} - \text{OD of test})}{\text{OD of control}} \times 100$$

OD of control= Absorbance of control (without extract).

OD of test= Absorbance of sample.

3. RESULTS

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3.1 Phytochemical Analysis

The results of phytochemical components qualitative screening in *phyllanthus emblica*, were listed in Table 1. Phallanthus emblica showed positive for phenol, Tannins,Flavonoids and Saponins which have medicinal properties.

Table1: Preliminary screening of phtochemicals compounds in *phyllanthus emblica*.

Compounds	Test	Results
Alkaloids	Mayer's Test	-
Phenols	Ferric Chloride Test	+
Tannins	Ferric Chloride Test	+
Flavonoids	Alkaline Reagent Test	+
Carbohydrates	Barfoed's Test	-
Saponins	Foam Test	+
Fixed oils and fats	Spot Test	-
Gums and Mucilage		-

3.2 HPLC Analysis

The HPLC analysis showed that 1 mg of ethanolic *phyllanthus emblica* fruit extract contains 64µg of Gallic acid.

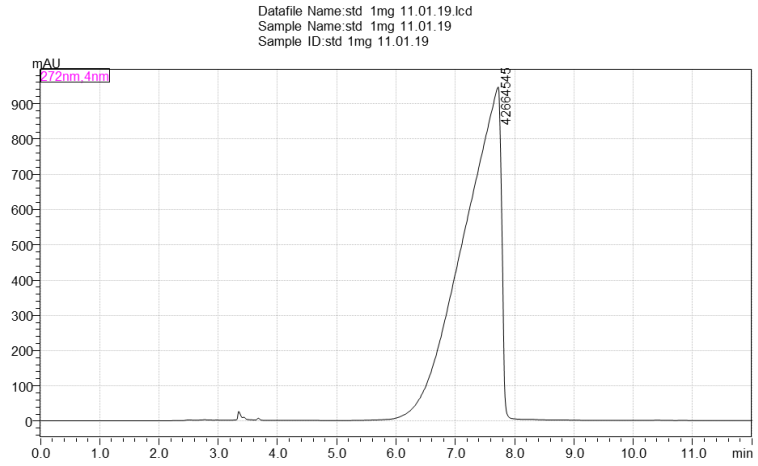

Fig.1 Standard Gallic acid (1mg/ml)

On X-axis = Retention time, On Y-axis = Measure of intensity of Absorbance

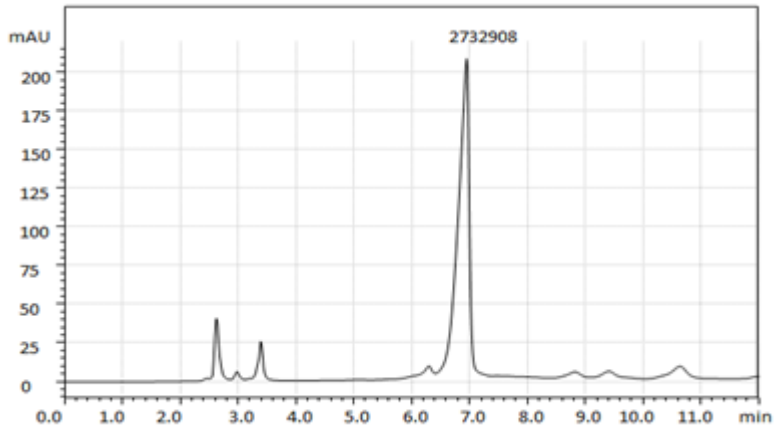

Fig 2. Ethanolic Extract of *Phyllanthus* fruit (1mg/ml)

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3.3 Determination of Total Phenolic Content

Phenolic compounds are the significant constituents of plants. These phenolic compounds are the dominant chain breaking antioxidants and may subsidise to antioxidant action.Results obtained in the present study showed that the toal phenolic content in the ethanolic extract of *Phyllanthus emblica* fruit extract was 1.14mg GAE/g and the total phenolic content was expressed as gallic acid equivalent.

3.4 Hydrogen peroxide radical scavenging activity

Phyllanthus emblica fruit extract antioxidant potential was investigated by hydrogen peroxide scavenging experiment. In this experiment, the extract was subjected to evaluate the ability of the extract to scavenge hydrogen peroxide radical and good scavenging activity was showed by the extract. The scavenging percentage and IC_{50} for the ethanolic extract of amla fruit were found to be 50 and 0.066µg/ml respectively. The scavenging percentage and IC_{50} value for the Ascorbic acid standard were found to be 50 and 0.24µg/ml.

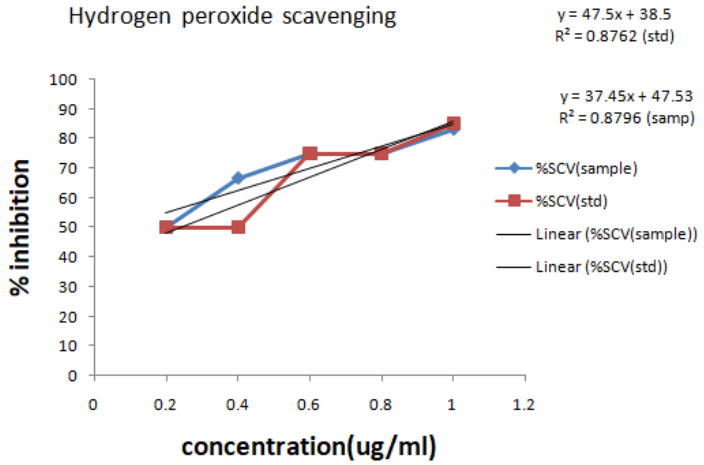

Fig 3. Hydrogen Peroxide Scavenging Assay

4. DISCUSSION

In the present study,Preliminary screening of phytochemicals revealed thepresence of important chemical constituents, like tannins, phenolic compounds, flavonoids and saponins. Ethanol was used as a solvent for the extraction of the amla fruit.Previous Investigations advocated that the presence of the phenolic compounds which satiate free radicals and prevents the oxidative damage to the body [31]. Oxidising agents such as hydrogen peroxide, reacts with Fe^{+2} by crossing the cell membrane which results in the formation of the hydroxyl radicals(free radicals). Hydrogen peroxide is capable of oxidising thiol groups and causes damage to the cells [32]. Several compounds were identified in amla fruit which have the cpability of antiproliferative activity against gastric and uterine cancer cells [33] and slow down the side effects of chemotherapy and radiotherapy [34-35]. Using gallic acid method, Total phenolics in the amla extract were estimated. Due to the presence of phenolic compounds, Our investigation suggested that the ethanolic amla fruit extract have the property which prevents the generation of the free radicals or eliminates the free radicals and this results in preventing damage to the cells.

5. CONCLUSION

In the present study, the ethanolic extract of *Phyllanthus emblica*fruits were screened for the presence of phytochemical compounds,for their total phenolic content and also for

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antioxidant activity. The results confirms the presence of phytochemicals and total phenolic compounds.The antioxidant potential of the ethanolic extract of the *phyllanthus emblica* may be due to the presence of the phytochemicals and the total phenol compounds.

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BENEFICIAL EFFECTS OF PHLOROGLUCINOL ON BEHAVIORAL IMPAIRMENTS IN ROTENONE INDUCED RAT MODEL OF PARKINSON'S DISEASE

Archana Imayavaramban[1] and Vijayalakshmi Krishnamurthy[2]
[1]Research Scholar, Department of Biochemistry Bharathi Women's College, Chennai.
[2]Associate Professor, Department of Biochemistry Bharathi Women's College, Chennai.

ABSTRACT: To investigate the behavioral characterizations of phloroglucinol on rotenone induced Parkinson's disease. Parkinson's disease was induced by intraperitoneal administration of 3mg/kg rotenone and a dose of 50mg/kg of phloroglucinol were administered orally to wistar albino rats. Behavioral studies such as Grip strength test, Hole board test, Hole cross test and Narrow beam test were conducted in all the animals. The rats injected with rotenone showed reasonable extent of Parkinsonism. In grip strength test, the muscle rigidity of PD induced animal seemed to be increased, when compared to control and the combination with phloroglucinol and L-dopa was more effective than the phloroglucinol. In hole board test, the exploratory behavior with rotenone showed significantly decrease in number of nose poking as compared to control and the combination with phloroglucinol and L-dopa has significantly increased the number of poking than the phloroglucinol alone. In hole cross test, the locomotor activity was significantly decreased in rotenone induced animal when compared to control and the combination with phloroglucinol and L-dopa showed increased locomotor activity. In narrow beam test, the motor coordination was decreased in rotenone induced animal when compared to control and the combination of Phloroglucinol and L-dopa shows increased motor coordination. This study indicates that phloroglucinol and L-dopa in combination has beneficial effects on the Parkinson induced animals.

KEY WORDS: Rotenone, exploratory behavior, hole cross, Narrow beam and phloroglucinol.

***Corresponding Author:** Dr. Vijayalakshmi Krishnamurthy
Associate Professor, Department of Biochemistry Bharathi Women's College, Chennai.
Email: viji42research@yahoo.co.in

1. INTRODUCTION

Neurodegenerationis described as a progressive loss of neuronal cell (Brain and Spinal cord). The most common neurodegenerative diseases include Parkinson's disease, Alzheimer's disease and Hungtington disease [1]. At present, there is no permanent treatment for parkinson's disease. It is a chronic and progressive neurodegenerative illness, due to the lack of dopamine and other neurotransmitters. The findings of PD is based on motor and non motor symptoms such as tremor, rigidity, bradykinesia, postural instability, dementia and memory loss [2]. It is the second neurological disorder after AD affecting 2-3% of the population above the age of 60 years.

There are several toxins available for inducing PD (MPTP, 6-OHDA, Reserpine and paraquat) and the rotenone depicts the classic features of PD [3]. Rotenone is commonly used as a pesticide and it is a mitochondrial toxin naturally occuring in plant species and it can easily cross the blood brain barrier. In 1985, rotenone was first used as a model for PD [4-5] reported that the rotenone when used for the chronic and systemic exposure leads to PD with behavioral features like hypokinesia and rigidity. Currently, a daily dose of rotenone through the intraperitoneal injection is considered as an ideal model of PD [6]. Many biochemical changes have been identified in the PD such as oxidative stress and damage to brain,

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mitochondrial dysfunction and evidence of inflammatory changes. Lack of pigmented dopaminergic neurons in the substantia nigra indicates the major neuropathological change in PD.

L-dopa is a dopamine precursor that is considered as a typical drug for the treatment of PD. Hence, L-dopa is used as the REFERENCES drug in the present study. Herbal products are used to treat Parkinson's disease in traditional medicine worldwide [7]. Phloroglucinol is a polyphenol that is a component of phlorotannins and belongs to the family Laminariaceae [8]. Previous studies have demonstrated that phloroglucinol has wide therapeutic effects that include, anti diabetic [9], antioxidant [10] and anticancer effects [11]. Previous literature reviews clearly evaluated the clinical evidence of Phloroglucinol in traditional medicines. In this study, the impact of phloroglucinol on behavioural studies in rotenone induced animal model of PD was examined.

2. MATERIALS AND METHODS

2.1 Chemicals

Phloroglucinol, Rotenone, L-dopa were procured from Sigma Aldrich. All the other chemicals used were of analytical grade.

2.2 Animals

Male wistar albino rats (150-250g) were used for the study. The protocol was reviewed and approved by the Institutional Animal Ethical Committee of Sathyabama University, Chennai (Registration number: SU/CLATR/IAEC/IX/067/2017). Animals were housed in cages (n=6 rats per cage) with husk bedding under standard conditions (24±2ºC, with 12h light/ dark cycle) and the rats were fed standard animal pellets and water *ad libitum*. The experiments were performed according to the guidelines of the Animal Ethics Committee, Government of India.

2.3 Experimental setup

Group – I: Control

Animals were administered with vehicle control (DMSO + Corn oil) for a period of 60 days.

Group – II: Rotenone treated

Animals in this group were administered with rotenone (3mg/kg body weight i.p) in DMSO: Corn oil for a period of 60 days.

Group – III: Rotenone + L-dopa treated

Animals in this group were administered with rotenone (3mg/kg body weight i.p) and simultaneously administered with L-dopa (10 mg/kg body weight p.o) daily for a period of 60 days.

Group – IV: Rotenone + Phloroglucinol treated

Animals in this group were administered with rotenone (3mg/kg body weight i.p) and simultaneously administered with Phloroglucinol (50 mg/kg body weight p.o) daily for a period of 60 days.

Group – V: Rotenone + L-dopa + Phloroglucinol treated

Animals were co-treated with Rotenone (3mg/kg body weight i.p), L-dopa (10 mg/kg body weight p.o) and Phloroglucinol (50 mg/kg body weight p.o) daily for a period of 60 days.

2.4 Measurement of body weight

The animals were weighed and body weight was evaluated before and after the treatment.

2.5 Behavioral assessments

2.5.1 Hole-board test

The hole board test was used to assess exploratory behavior in wistar albino rats. The board having a size of 40×40 cm, totally 16 holes are dispersed evenly on the floor and each hole

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contains 3cm in diameter. Each rat was skilled to insert its head into holes. Subsequently, the animals were allowed to explore the hole for 5mins and the number of pokes per 5mins was observed and quantified by the method of [12].

2.5.2 Hole - crossing test

Hole crossing activity was used to determine the locomotor activity by the method of [13]. The partition was fixed in the middle of the cage that contains 30×20×14 cm. A hole was made in the partition at a height of 7.5 cm from the bottom of the cage. Control, induced and treated group rats were placed in the cage and allowed to cross the hole. The number of crossing was recorded for 3mins.

2.5.3 Narrow beam test

Narrow beam test is used to describe the motor coordination ability of the rodents. The animals were allowed to walk across a narrow wooden beam that consist of two platforms connected by a wooden beam (0.5 mm in thickness, 2.0 cm in width and 120 cm in length). The beam was made up to 50cm height over the ground. For protection saw-dust was placed under the beam. Before the experiment the rats were allowed to explore and they are practiced to walk on the narrow wooden beam from one end to other end. The number of falling and time taken to cross the beam was recorded [14].

2.5.4 Grip Strength Test

Grip strength was used to determine the muscle weakness. The animals were released to hang on with its forelimbs, while its body and tail was balanced in the air above 50cm from the bottom of the ground. The time taken to cling on the rope was noted [15-16].

3. RESULTS

3.1 Body weight of experimental rats

Rotenone treated rats showed a gradual decrease in body weight during the course of treatment and significant decrease was recorded. Treatment with phloroglucinol along with rotenone, L-dopa with rotenone and the combination of L-dopa, Phloroglucinol and rotenone significantly ($p< 0.001$) prevented the reduction in body weight as compared with the rotenone treated group (Figure. 1).

3.2 Exploratory behavior

Animals were treated with rotenone showed significant ($p< 0.001$) reduction in number of nose poking as compared to vehicle control. The combination of L-dopa and phloroglucinol along with rotenone significantly ($p< 0.001$) increased number of poking as compared to rotenone treated group. Number of poking was also increased in Phloroglucinol, L-dopa treated group ($p< 0.01$) (Figure. 2).

3.3 Locomotor activity

Locomotor activity of rats in rotenone treated group was significantly ($p< 0.001$) reduced as compared to vehicle control. The combination of L-dopa and Phloroglucinol along with rotenone significantly ($p< 0.001$) increases the locomotor activity as compared to rotenone treated group. L-dopa, Phloroglucinol treated group has significantly ($p<0.01$) increased the locomotor activity. (Figure. 3).

3.4 Motor co-ordination

Rotenone treated rats showed a gradual decrease in motor co-ordination and significantly showed ($p<0.001$) increase in time to cross the beam and number of foot slip as compared to vehicle control. Treatment with the combination of L-dopa, phloroglucinol with rotenone significantly ($p<0.001$) attenuated the time to cross the beam and number of foot slip as compared with the rotenone treated group. L-dopa, Phloroglucinol treated group significantly ($p< 0.01$) increases the motor co-ordination (Figure. 4 - 5).

3.5 Muscular Strength

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Rotenone treated animals showed significant ($p<0.001$) reduction of grip strength which could be due to a loss of muscular strength as compared to vehicle control. Treatment with L-dopa and phloroglucinol showed significant ($p< 0.001$) improvement in the muscular strength as there is an increase in retention time and fall of time in grip strength test. L-dopa, Phloroglucinol treated group showed increased muscular strength ($p<0.01$) (Figure. 6).

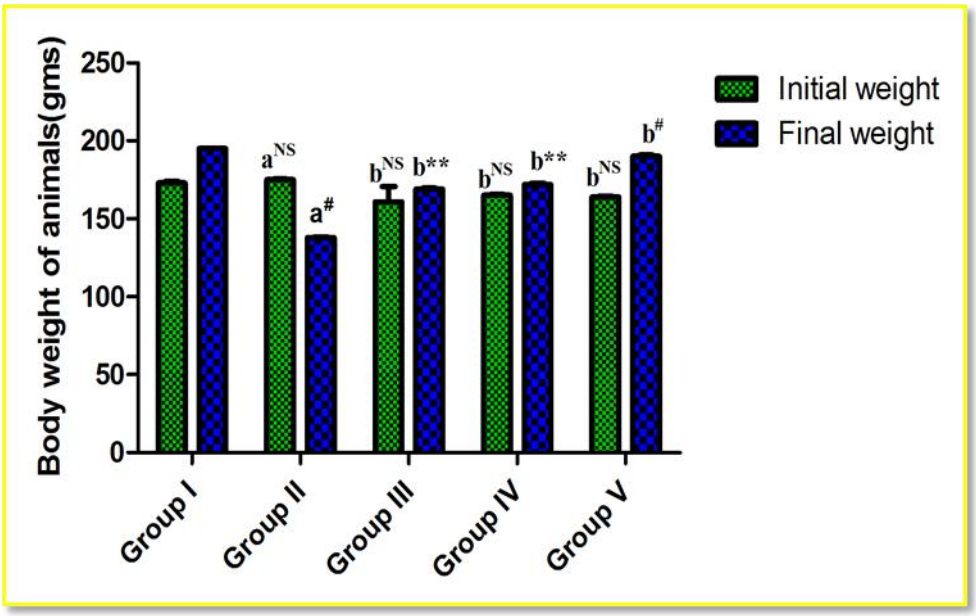

Figure 1: ***The effects of phloroglucinol on body weight of experimental rats***. Statistical significance # $p< 0.001$, **$p< 0.01$, *$p< 0.05$, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

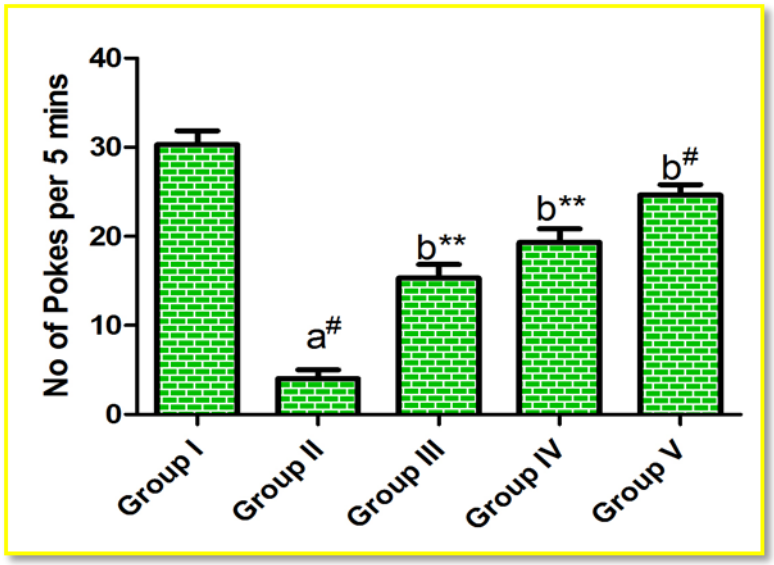

Figure 2: Effect of phloroglucinol on exploratory behavior (Hole board test) in rotenone treated rats. All values are expressed as mean ± SD (n = 6). Statistical significance # $p< 0.001$, **$p< 0.01$, *$p< 0.05$, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

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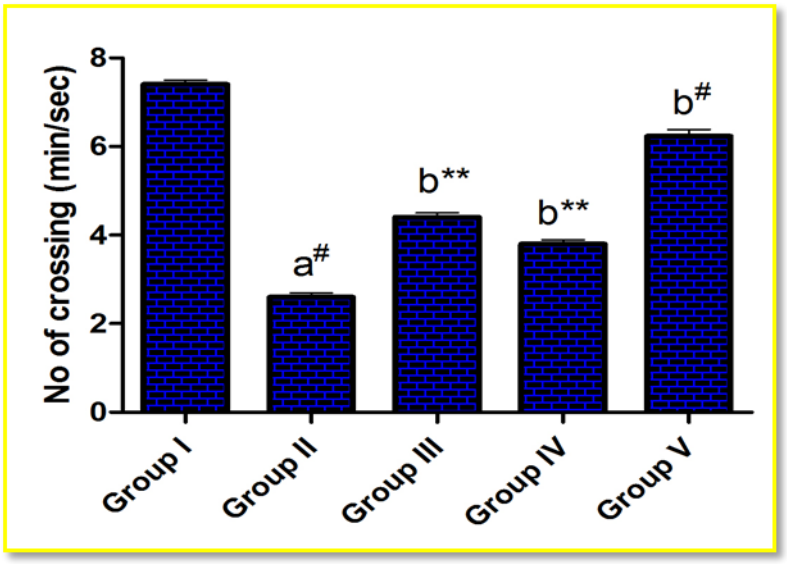

Figure 3: Effect of phloroglucinol on locomotor activity (hole cross test) in rotenone treated rats. All values are expressed as mean ± SD (n = 6). Statistical significance # p< 0.001, **p< 0.01, *p< 0.05, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

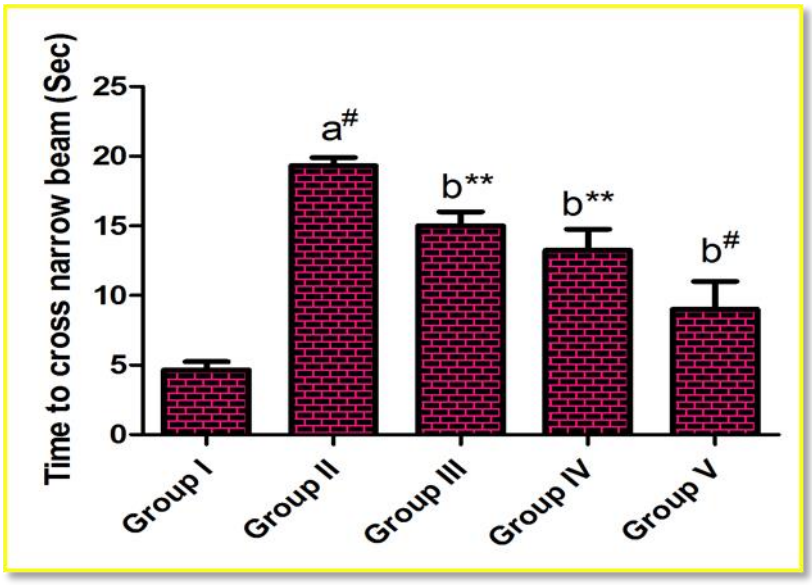

Figure 4: Effect of phloroglucinol on motor coordination (narrow beam test – transfer latency) in rotenone treated rats. All values are expressed as mean ± SD (n = 6). Statistical significance # p< 0.001, **p< 0.01, *p< 0.05, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

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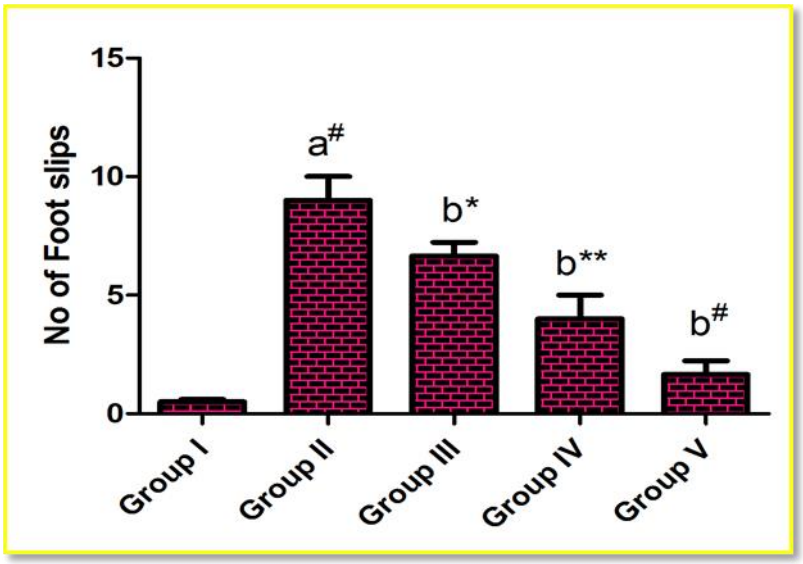

Figure 5: Effect of phloroglucinol on motor coordination (narrow beam test – No of foot slip) in rotenone treated rats. All values are expressed as mean ± SD (n = 6). Statistical significance # p< 0.001, **p< 0.01, *p< 0.05, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

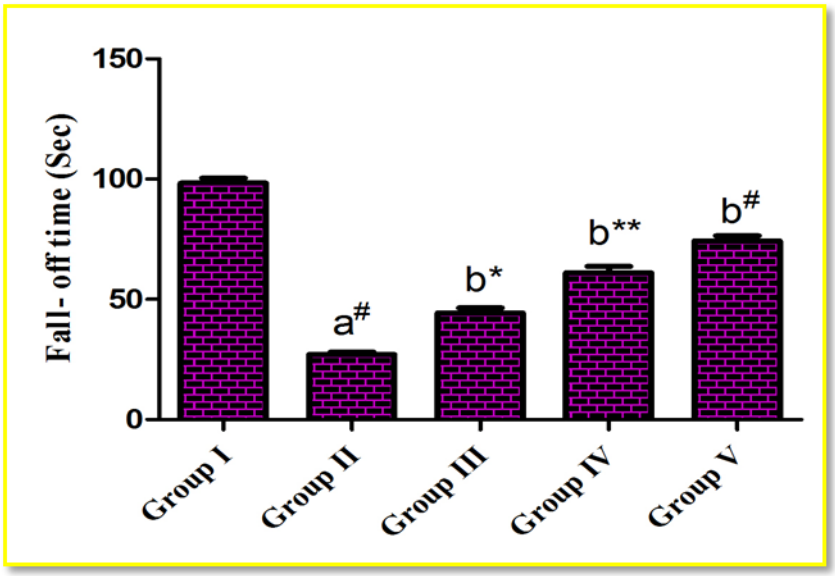

Figure 6: Effect of phloroglucinol on muscle weakness (grip strength test) in rotenone treated rats.All values are expressed as mean ± SD (n = 6). Statistical significance # p< 0.001, **p< 0.01, *p< 0.05, NS – Non significant. a – Comparison between group II and group I. b – Comparison between group III, IV, V and group II.

4. DISCUSSION

The rotenone induced PD model is associated with neuronal damage in the Substantial nigra and striatum, which can manifest as marked deterioration in motor function, behavioral changes, loss of body weight and altered muscle morphology [17]. Variety of behavioral task where loss of dopaminergic function resulted in decreased activities such as locomotion [18], total distance travelled [19] and Fore limb use [20-21]. This finding indicates that the neuro-

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behavioral effect of Phloroglucinol have been evaluated through hole board test, hole crossing test, Narrow beam test and grip strength test on wistar albino rats.

Hole board test demonstrated that head dipping behavior is sensitive to change in emotional state of the animal and suggested that the expression of an anxiolytic state in animals as reflected by an increase in head poking behavior [22]. In our study, Phloroglucinol increased head dip counts without changing locomotion in the hole-board test when compared to rotenone induced wistar albino rats. The hole board board test is used to identify the anxiety in animals and in this test an anxiolytic like state may be reflected by an increasing in head dipping behaviors [22].Gama-amino butyric acid (GABA) is a major inhibitory neurotransmitter in the CNS [23]. Different types of anxiolytics, muscle relexant; sedative-hypnotic drugs have shown their action through GABA. This type of effect is analyzed with the drugs that act on GABA/Benzodiazepine receptor complex. Most of the anxiolytic agents exert their action by opening of activated GABA-chloride channel [24]. The rotenone treated rat shows less exploratory behavior and decreased abilitiy to maintain balance which was observed by hole board test [25]. The Nebivolol was significantly increases the exploratory behavior as compared to rotenone treated group was observed in hole board test [26].

The study on locomotor acitivity was evaluated by hole cross test. The activity was measured by the level of excitability of the CNS and this decrease may be closely related to sedation resulting from depression of the Central nervous system [27]. Phloroglucinol shows significant increase in the locomotor activity compared to rotenone induced wistar albino rats. [28] Reported that *Dhatri Lauha* demonstrates insignificant changes in behavior related to CNS activities in mice. [29] Shows that diclofenac significantly increases the number of crossing compared with chlorpromazine induced rats. [30] State that *Cleome rutidosperma* gradually increases the number of crossing was observed in the hole cross test.

Narrow beam test was used to assess motor coordination and it is first described by [31]. It consists of accessing the rodent's ability to traverse a graded series of narrow beams to reach the other end of the platform. The beam is made up of wooden square strips with the size of 3.5cm in diameter and 200cm long [32-33]. The test was modified by fitting a step-down ledge that prevents falling of rats [34]. In our study, motor activity by the narrow beam test required minimal training for maximal performance; decreased the need for extensive training and reliability of the data [35]. It is used to test the balance, vestibular integrity and muscular co-ordination[36]. Similar finding was described by γ-tocopherol treated against MPTP induced Parkinsonic mice [37]. The beneficial effect of antidepressants against rotenone induced Parkinsonism like symptoms by narrow beam test [38].

Muscle weakness is assessed by grip strength test. It is a common symptom in Parkinson's and several studies have reported weakness in people with parkinson's disease [39]. Grip strength test shows a decrease in muscle coordination which could be due to the loss of muscular strength. Treatment with phloroglucinol shows an increase in the fall-off time when compared to rotenone group[40]. described that Methanolic extract of *Canscora decussata* improved the fall of time when compared with MPTP treated mice was observe in the grip strength test [41]. Reported that the *Ganoderma lucidum* significantly increases the fall off time and decreases in MPTP treated swiss albino mice by grip strength test. The effect of hesperidin on behavioral activities in 6-OHDA induced animal shows decreased fall of time in the grip strength test [42].

5. CONCLUSION

Based on the above study, it may be concluded that the combination of Phloroglucinol with L-dopa attenuates rotenone induced behavioral impairments in wistar albino rat model of PD.

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Further studies on gene expression may through light its mechanism and it may act as a novel and promising drug for the management of PD.

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IN VITRO STUDY OF THE ANTI-INFLAMMATORY ACTIVITY OF QUERCETIN FROM *MURRAYA KOENIGII* LEAF EXTRACT

H N Deepa[1], Varsha K S[1], Akhsa K Alexander[1], Jayashree S[1]
[1]School of Biochemistry, REVA University, Bangalore – 560064, India.

ABSTRACT: *Murraya koeingii* (curry leaves) is a medicinal herb and has been widely used in Asian countries for the treatment of diabetics and hypertension. *M. koenigii* leaves are commonly used in snacks, food item and in regular foods for taste and flavor. Moreover, their leaves have numerous pharmaceutical properties such as antioxidant, anti-inflammatory and anti-cancerous activities. In the present study, *M. koenigii* leaves were extracted with ethanol by using soxhlet method of extraction and evaluated for anti-inflammatory activity on inflammatory cell line RAW 264.7.Through the HPLC analysis, it was found the presence of quercetin in the ethanolic extract of curry leaves. The radical scavenging effect in *M. koenigii* was observed with IC50=72.56µg/ml inhibition on RAW 264.7 cell line. Here, the target compound was Quercetin, a flavonoid isolated from *M. koenigii* leaves by column chromatography method and HPLC analysis to confirm the presence of Quercetin compound. These findings strongly suggested that, Quercetin shows significant effects in anti-inflammatory activity and there is a potential for Quercetin to be further investigated for its applicability in treating inflammatory disorders.

KEYWORDS: Anti-inflammatory, *Murraya koenigii*, Quercetin, RAW 264.7 cells

***Corresponding author**: H N Deepa
School of Biochemistry, REVA University, Bangalore, India.
Email: deepahn@reva.edu.in

1. INTRODUCTION

After decades of serious obsession with the modern medicinal system, people have started looking at the ancient healing systems like Ayurveda, Siddha and Unani. This is because of the adverse effects associated with the synthetic drugs. Ancient Indian literature incorporates a remarkably broad definition of medicinal plants and considers all herbal plants to be potential sources of medicinal substance [1]. Curry leaves (*Murraya koenigii*), belonging to the family Rutacea, is found in India, Srilanka, Bangladesh and other South Asian countries [2]. It is commonly used in cooking and also widely used in Indian traditional medicine for thousands of years [3]. Traditionally, the plant is used as stimulant, stomachic, analgesic and for the treatment of diarrhea, dysentery [4]. Previous phytochemical investigation on this plant revealed Antidiarrhoea, Anti-bacterial, Anti-fungal and Hypoglycemic activities [3, 4, 5].

Inflammation is a physiologic series of responsesgenerated by the host in response to infections and other insults. Inflammation can have a rapid onset and last a short period of time (acute inflammation) or it can persist due to a continuous stimulus or injury (chronic inflammation). The initial events of the inflammation are derived from vascular reactions at the site of the injury. Vascular changes are important for the induction of the response and are characterized by redness, heat and swelling, usually accompanied by pain and loss of function and collectively represents the “cardinal signs” of inflammation. These signs of inflammation are the results of vasodilation and increased vascular permeability, leading to exudation of fluid and plasma proteins and recruitment of leukocytes to the site of injury [6]. Quercetin, a plant flavonoid found in many plant kingdom, including many edible species. Flavonoids are an important group of plant secondary metabolites with a wide range of biological activities [7]. It is most widely noticed in common beverages like tea, beer and

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wine. Quercetin is an eosinophilic inflammation suppressor [8]. It fights against the inflammation and prevents the cardiovascular diseases [9]. It exhibits important vasorelaxant properties on isolated arteries which helps to lower blood pressure and prevents the development of cardiac hypertrophy [10, 11].

Quercetin have an inhibitory effect on the proliferative effectiveness on the cellular migration and on the accumulation of the collagen in the injured sites. It has a significant role in acute and chronic inflammation [12]. When an infection is caused due to the virus, bacteria the body tries to heal itself. One of the core most remarkable properties of quercetin is its ability to modulate inflammation. It inhibits inflammatory enzymes cyclooxygenase (COX) and lipooxygenases thereby, decreasing inflammatory mediators such as prostaglandins and leukotrienes [13, 14]. Treatment of inflammation with Non-steroidal anti-inflammatory drugs might cause adverse drug reactions. Quercetin might exhibit greater effect on inflammation if used in medicines. The search for naturally occurring quercetin has a great interest in industries as well as in scientific research [15].

The present study mainly focuses on the isolation of Quercetin from *M.koenigii* by column chromatography and HPLC analysis and the future study would be to test the isolated quercetin compound on inflammatory RAW 264.7 cell line.

2. MATERIALS AND METHODS

2.1 Sample collection

Fresh curry leaves were collected from nearby places of Bangalore and kept for shade drying.

2.2 Extraction

Leaves were shade dried for 3 weeks later it was grinded to powder. The powder was used for ethanolic extract using soxhlet apparatus. The extracts were later filtered using Whatman's No.1 filter paper. After the extraction, the solvent was distilled off using rotary evaporator and sample was dried. The extracts thus obtained were dissolved in ethanol at the concentration of 1mg/ml for further analysis [16].

2.3 Preliminary test for phytochemicals

The preliminary test for phytochemicals from ethanolic extracts of plant powder of curry leaves were carried out to find the presence of totally different secondary metabolites (phytochemical constituents) such as alkaloids, flavonoids, tannins, phenols and terpenoids [16].

2.4 Nitric oxide scavenging assay

Nitric oxide (NO) is a short-lived free radical that mediates many biological processes. One of the functions of NO is to enhance the bactericidal and tumoricidal activities of activated macrophages. Excessive production of NO could however potentially lead to tissue damage and activation of pro-inflammatory mediators.

The Murine monocytic macrophage RAW 264.7 cell line was cultured in Dulbecco's Modified Eagle Media (DMEM) (2Mml- glutamine, 45g/l glucose, 1Mm Sodium pyruvate) with 10% fetal bovine serum (FBS). The cells were cultured at 37° c with 5% CO2and were sub cultured twice a week. The cells were seeded in 96- well tissue culture plates ($1X10^6$ cells / ml) and incubate fir 24h at 37°c with 5% CO2. Then 100µl test extract in Dimethyl sulfoxide was serially diluted to give a two- fold concentration variations and then added on to cells. Cells were then stimulated with and 10 µg/ml *E. coli* lipopolysaccharide (LPS) and incubated at 37°c for another 17h. After 24h incubation, 100µl of supernatant from each well of cell culture plates was transferred in to 96- well micro titer plates and equal volume of Griess reagent was added. The absorbance of the resultant solutions in the well of the micro titer plate was determined with a micro titer plate reader (Tec can device) after 10 min at 550nm. The concentration of nitrite were calculated from regression analysis using serial

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dilutions of sodium nitrite as a standard. Percentage inhibition was calculated based on the ability of extracts to inhibit nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0% inhibition [17].

2.5 Column chromatography

10g of ethanol leaf extract were chromatographed over silica gel column using solvents with an increasing polarity. The mixture was packed on silica gel column and elution starts with 100% Hexane and then the increased polarity using Chloroform, Ethyl acetate, Ethanol and Methanol in the ratio 45:5, 40:10, 35:15, 25:25.The fractions obtained were then combined and solvents were made to evaporate [18]. Once the solvents gets evaporated, the extract was dried and weighed. The average yield obtained was 1.02g.

2.6 HPLC analysis

The dried leaves of *M. koenigii* was grinded to obtain a coarse powder. It was then extracted using soxhlet extraction with ethanol [19].

2.6.1 Preparation of Standard stock solution

25mg of standard quercetin was weighed and was transferred to 25ml of volumetric flask and dissolved in methanol to obtain 1000µg/ml.

2.6.2 Preparation of Sample stock solution

25mg of sample quercetin was weighed and was transferred to 25ml of volumetric flask and dissolved in methanol to obtain 1000µg/ml.

Standard and the isolated fraction of quercetin were analyzed by HPLC technique using the following conditions.

Parameters	**Description**
Column	HiQ Sil C18HS
Column size	4.6mm*250mm*5µ
Mobile phase	Methanol: 0.1%ortho phosphoric Acid (65:35%)
Flow rate	1ml/min
Detectors and wavelength	UV detector, 369nm
Injection loop capacity	10µl
Concentration of Samples	10 pm (standard and isolated fraction of quercetin)
Retention time	8.4min
Run time	13min

3. RESULTS

3.1 Nitric oxide scavenging assay

NO scavenging assay of plant extract were performed by the amendment in RAW 264.7 cell line culture. Different concentration of plant extract showed the different enzyme actvity inhibition as dipiected in table 1 and figure 1. After treatment showed significant dose dependent inhibition of growth of RAW 264.7 cells at IC50 values of 72.56 µg/ml.Thus,this results suggests that plant extracts show significant level of inhibition of RAW 264.7 cell

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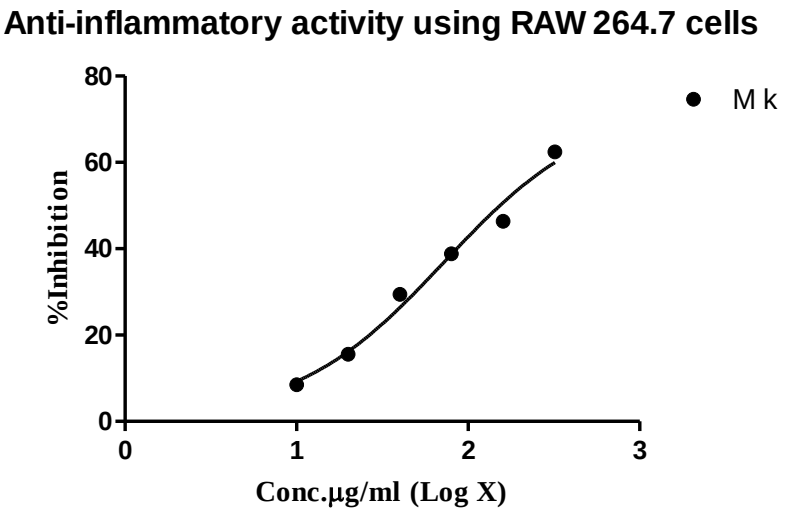

	M k
LogIC50	1.861
IC50	72.56

Figure 1: Nitric oxide scavenging assay of *M. koenigii*

Compound name	**Conc. µg/ml**	**OD at 590nm**	**% Inhibition**	**IC50 µg/mL**
Control	0	0.7551	0.00	
M k	10	0.6910	8.49	
	20	0.6375	15.57	
	40	0.5326	29.46	**72.56**
	80	0.4619	38.83	
	160	0.4049	46.38	
	320	0.2835	62.46	

Table 1. IC50 of M. koeigii plant extract

3.2 HPLC Analysis of Quercetin

The *Murraya koenigii* leaf extract was evaluated for presence of Quercetin, one of the important flavonoids present in plant by HPLC. The Quercetin standard eluted at retention time of 3.48 min (Fig. 3). The HPLC analysis of *M. koenigii* leaf extract showed the presence of Quercetin (Fig. 4). The area of standard Quercetin was compared with that of sample and Quercetin content was estimated to be 1.15 ug/g of extract.

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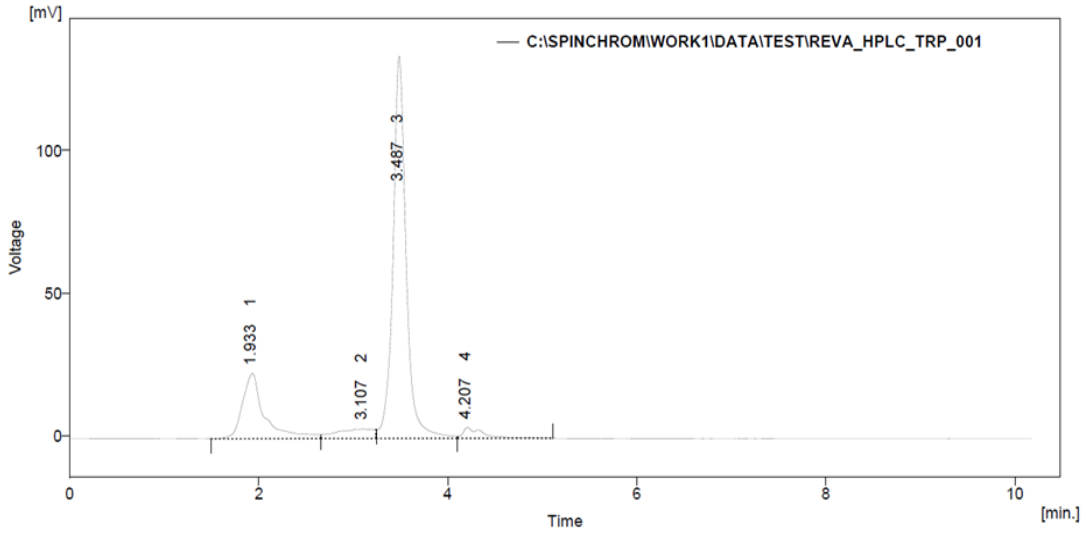
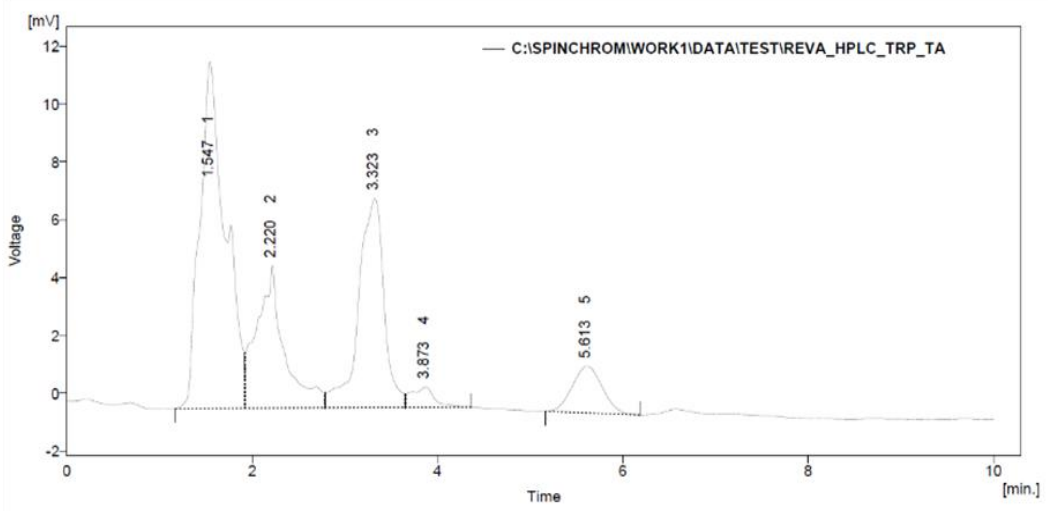


Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\TEST\REVA_HPLC_TRP_001)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.933	373.177	22.881	20.5	14.0	0.20
2	3.107	92.433	3.352	5.1	2.0	0.49
3	3.487	1296.195	133.916	71.3	81.6	0.14
4	4.207	55.054	3.869	3.0	2.4	0.22
	Total	1816.859	164.018	100.0	100.0	

Figure 2: HPLC graph for Standard



Result Table (Uncal - C:\SPINCHROM\WORK1\DATA\TEST\REVA_HPLC_TRP_TA)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.547	229.640	11.970	44.3	45.3	0.27
2	2.220	101.600	4.922	19.6	18.6	0.28
3	3.323	138.804	7.220	26.8	27.3	0.28
4	3.873	12.358	0.706	2.4	2.7	0.30
5	5.613	36.406	1.612	7.0	6.1	0.36
	Total	518.808	26.429	100.0	100.0	

Figure 3: HPLC graph for Sample

4. DISCUSSION

Murraya koenigii is used as a flavoring agent. Traditionally, it is also used in alternative

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forms of medicine to treat many ailments. It is rich in polyphenols/ flavonoids which are known to possess chemo-preventive and chemo-therapeutics potential [20]. *M.koenigii* leaves have been reported to contain flavonoids such as quercetin, rutin, myricitin. Quercetin is found to possess many pharmacological applications and have been studied for their pharmacological properties in the recent years [21]. In this context, quercetin was isolated from ethanolic extract of *M. koenigii* leaves through column chromatography and HPLC. Nitric oxide scavenging assay was carried out and the effect of *M. koenigii* was observed with IC 50 value of 72.56µg/ml inhibition on RAW 264.7 cell line. Column chromatography was carried out using four different solvents with increasing polarity and the final yield obtained was 1.02g. HPLC graph has revealed the presence of quercetin compound in the ethanolic extract of curry leaves.

5. CONCLUSION

The current study adds insight about the in vitro assessment of quercetin on inflammation. This study has revealed that quercetin has an anti-inflammatory properties. Hence, there is a need to investigate the potential application of quercetin and the research must also focus on the studies that improve the quercetin effectiveness in order to benefit the humanity from their inherent the therapeutic potential. Future research of quercetin on inflammatory RAW 264.7 cell line will be necessary to determine its inflammatory activity in treating any form of inflammatory diseases.

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QUALITATIVE AND QUANTITATIVE ASPECT OF VARIOUS SECONDARY METABOLITES PRESENT IN *TRIGONELLA FOENUM-GRAECUM* LEAVES

Ramesh Kumar Kushwaha[1*], Risha Das[1], Ankita Debnath[1], Budhaditya Dey[1]
[1]Department of Biochemistry, School of Applied Sciences, REVA University, Bangalore-64,

ABSTRACT: *Trigonella foenum-graecum,* commonly called as fenugreek, is known for its culinary and medicinal uses. Among the spices, fenugreek is used as an esoteric food adjacent to enhance the flavour and colour of the food. The leaves of the plant are commonly used as vegetables because they are a rich source of calcium, iron, beta-carotene, and other phytochemicals. The properties it possesses, includes anti-cancerous, anti-inflammatory, anti-septic and, anti-helminthic.*T. foenum-graecum* is known for the strong hypoglycemic effects of the seed extract and for its significant allelopathic effects. The leaf extract however has not been studied thoroughly up-to-date and very less data are available regarding the quantitative content of the plant's leaves. The purpose of this study was to determine the qualitative and quantitative analysis of the various secondary metabolites (alkaloids, terpenoids, flavonoids, tannins and phenols)by using an HPLC (High Performance Liquid Chromatography) method. The plant leaves were extracted in methanol by using soxhlet extraction process, followed by a rotary evaporator, to get the fine-powdered form. Qualitative test was then performed, and suggested the presence of few secondary metabolites in the fenugreek leaves extract. On performing the quantitative analysis using HPLC, a high content of a polyphenolic metabolite called gallic acid was found in the methanolic extract of the fenugreek leaves. Further, the anti-oxidant activity was evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay. These results suggested that methanolic extract of fenugreek leaf is not only an important source of secondary metabolites but also a potential source of dietary polyphenolic antioxidants.

KEYWORDS:*Trigonellafoenum-graecum;* HPLC; antioxidants; phenols; gallic acid.

***Corresponding author:** Ramesh Kumar Kushwaha
Department of Biochemistry, School of Applied Sciences, REVA University, Bangalore, Karnataka, Email Address: kush0109@yahoo.com

1. INTRODUCTION

Plant-derived natural products have long-standing utility toward treating degenerative diseases. Plants have survived for millions of years on planet earth by continuously evolving and adapting [1]. They gained the ability to develop defense mechanisms by biosynthesizing metabolites as a guard against the external factors, such as pests and climatic conditions [2]. Undoubtedly, it is known that herbal medicine is one of the oldest forms of health care. Even today, according to the World Health Organization (WHO), it is estimated that 80% of the world's population still relies primarily on botanical medicines [3]. Medicinal plants constitute the main source of new pharmaceuticals and healthcare products [4]. The utilisation of medicinal plants in the industrialised societies has been traced for the extraction and development of various drugs from these plants and from medicines which has been traditionally used [5]. Extraction and characterization of various active phytocompounds from these medicinal plants has given birth to some high activity profile drugs [6]. It is evident that secondary plant metabolites play crucial roles in human health and may be nutritionally important [7].

Fenugreek (*Trigonella foenum-graecum*), is a short-living annual plant, and belongs to

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the Fabaceae family. It is grown in many parts of Asia, Africa, and Europe as food, condiment, spice, and as native medicine [8,9]. The genus *Trigonella* is named in REFERENCES to its triangular shaped flowers, and in Latin little triangle is referred to as *Trigonella* [10]. The species *foenum- graecum* gets its name from historical perspective of Romans, since referred to it as Greek-hay, symbolizing it as the common crop used as fodder for animals in Greece [11].

Fenugreek is used as a spice and herb in many culinary dishes and its green leaves and seeds are the only parts of the plant that are edible [12]. In Indian cuisine, its leaves are used to flavor dishes or eaten as greens, and its seeds are used for seasonings or are crushed for the preparation of curry powder and pastes [13]. Not only it is used in various food preparations, it also has healing benefits. It is one of the oldest medicinal plant and its medicinal properties are well documented in the ancient medical literature [10]. Apart from its medicinal values, its other important uses are nitrogen fixation in the soil, feedstock of the food and chemical industry and livestock feed [10,14]. Among the beneficial physiological effects, the anti-diabetic and hypocholesterolemic property of this plant characterizes the intrinsic dietary fiber constituent, having favorable neutraceutical value [13].

This plant is an important source of biologically active compounds like alkaloids, polyphenols, tannins, terpenoids, vitamins and essential oils, which can be extracted and isolated using various organic solvents. The seeds of this plant contain a rich source of bioactive antioxidant substances, and are used mostly in food preparations and herbal formulations. Numerous reports have shown that these compounds contain various properties which are useful in food, cosmetics, and pharmaceutical industries [15]. The anti-diabetic effect of fenugreek helps type I diabetic patient to lower the lipid peroxidation when taken as supplement in the diet and are helpful in digestive disorders such as dysentery, diarrhea, and flatulence. It is an important source, possessing antioxidant property, which is known to give protection against reactive oxygen species (ROS) induced damage, acting as radical scavenging and prevention of damage caused to cellular components like membranes [16].

Reports suggested a direct relationship between the phenolic content and the anti-oxidant activity. Fenugreek is considered to be a potential source of anti-oxidant compound, due to the presence of high content of phenols like gallic acid. The phenolic content tends to reduce the risk of heart diseases. Anti-oxidant based drug designs have been used for the prevention and treatment of intricate diseases like atherosclerosis, stroke, diabetes, cancer, and Alzheimer's disease [17]. Various oxidative studies have reported the imbalance between reactive oxygen species and antioxidant defense systems, which might increase the oxidative stress and cause damage to macromolecules such as carbohydrate, DNA, proteins. Currently, a large number of studies have shed positive light on fenugreek's medicinal properties, such as anti-oxidant [16], anti-inflammatory [18], anti-diabetic [19], anti-obesity [20], anti-cancer [21], hepatoprotective [22], anti-hyperlipidemic [23], women's health[24] and sexual health modulating activities [25]. The presence of high quantity of iron in fenugreek is favorable in treating anaemia[26].

With this line of background, the present investigation was designed to assess the free radical scavenging ability as well as the qualitative and quantitative aspect of various secondary metabolites present in the methanolic extract of leaves of fenugreek plant.

2. MATERIALS AND METHODS

2.1 Plant material collection and preparation of the extract

Samples (leaves) of the plant *T. foenum-graecum* were collected from the local markets of Bangalore, Karnataka. The collected plant samples were washed under running tap water for 4-5 times, and dried under sun. The dried mass was blended into fine powder by frequent

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sieving followed by grinding. This powder was extracted by Soxhlet process with 100% methanol (70mL). The process was carried out two times to check maximum extraction. After extraction, the content was concentrated at maintained conditions and filtered through a Whattman filter paper and the extracted solvent was evaporated at 40ºC in a vacuum rotary evaporator to get the corresponding powdered form of the extract which was stored at -4ºC and used for further qualitative analysis.

2.2 Preliminary Tests for Phytochemicals

The preliminary test for phytochemicals from methanolic extracts of leaf powder of fenugreekwere carried out in order to find the presence of various secondary metabolites (phytochemical constituents) like alkaloids, flavonoids, terpenoids, phenols and tannins.

2.2.1 Test for Alkaloid (Mayer's test):

To 1mL of fenugreek leaf extract, 1mL of Mayer's reagent and few drops of iodine solution were taken in a test tube. Formation of yellow precipitate indicates the presence of alkaloid.

2.2.2 Test for Flavonoids:

1mL of 10% lead acetate solution was added to a pinch of crude extract. Formation of yellow colour indicates the presence of flavonoid.

2.2.3 Test for Terpenoids:

In 2mLof chloroform, a little amount of crude sample was suspended and evaporated to dryness. Then 2mL of conc. sulphuric acid was added and solution was heated for 2 min. Formation of greyish-black colour indicates the presence of terpenoid.

2.2.4 Test for Phenols and Tannins:

A pinch of crude extract was mixed with 2mL of distilled water, and few drops of $FeCl_3$ Solution were added. The solution was shaken or stirred properly. Development of green precipitate and greenish-blue colour confirms the presence of tannins and phenols respectively.

2.3 DPPH radical scavenging assay

The DPPH scavenging assay was a modification of the procedure of Moon and Terao*(1998)* [27]. Briefly, 5mg, 10mg, 20mg, 30mg, 50mg, and 100mg of dried sample were weighed and then ground and sieved respectively, followed by methanolic extraction. 100µL from each concentration were taken in different test tubes, followed by the addition of Tris-HCl (900µL), and DDPH(2mL). A blank solute was prepared, where 100µL of methanol was used instead of plant extract followed by the addition of Tris-HCL(900µL), and DPPH (2mL). All the test tubes were incubated at 37°C for 30 minutes.The resultant absorbance was recorded at 517nm.

2.4 HPLC (High Performance Liquid Chromatography) analysis

The dried extracts were used and dissolved in HPLC grade methanol, which was then filtered and subjected to quantitative analysis by means of a Shimadzu LC-20AD (Kyoto, Japan) HPLC instrument. The equipment of the instrument includes dual-pump LC-20AD binary system (Shimadzu) HPLC, Phenomenex Luna C-18 (250 × 4.6 mm, 5µm) column, a Prominence system controller CBM 20A Lite, Prominence Photodiode Array detector SPD-M20A (Shimadzu) and a manual injector with 100µL syringe and adaptar plate. Data were integrated by using Shimadzu class VP series software. The standard phytochemical used for the HPLC analysis was Gallic acid.Separation was achieved with acetonitrile containing 1% acetic acid in a linear gradient program, for a time interval of 20 minutes. Results were obtained by comparison of peak areas (λmax =254 nm) of the samples (400 mg dry extract) with that of standards 20 µg/mL [26]. The solvent gradient in volumetric ratios of solvents A and B was as follows: 0-10min, 60A/40B; 10-15min, 40A/60B; 15-20min, 20A/80B. Dual Wavelength of 272nm and 254nm were used to detect the eluent [16]. The elution was completed at a flow rate of 1.0mL/min.

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3. RESULTS

3.1 Phytochemical analysis

The analysis of the extract of fenugreek, revealed the presence of various phytochemicalspossessing properties useful in herbal medicines. From *Table 1*, it was seen that phenols, tannins, terpenoids, and flavonoids were present in the methanolic extract. However, alkaloids were not observed, and can be concluded to be absent in the studied extract. Studies have suggested that phenolic compound contributes to the majority of anti-oxidant activity. Thus, plants possessinghigh quantity of phenolic compounds are a good source of natural anti-oxidant.

Table 1: Preliminary screening of phtochemical compounds in fenugreek leaves extract.

Phytochemicals	Results
Alkaloids	-
Flavonoids	+
Terpenoids	+
Phenols and tannins	+

(KEY: '+' = presence; '-' = absence.)

3.2 HPLC (High Performance Liquid Chromatography) analysis

The extract of fenugreek leaves was analyzedto determine the phenolic content by biochemical methods. HPLC analysis revealed that the extract contains gallic acid in high amount, which was confirmed by spiking the extract with that of the standard. Total gallic acid in the extract was found to be 544mg/g, where the concentration of the sample taken was 10µg/mL.

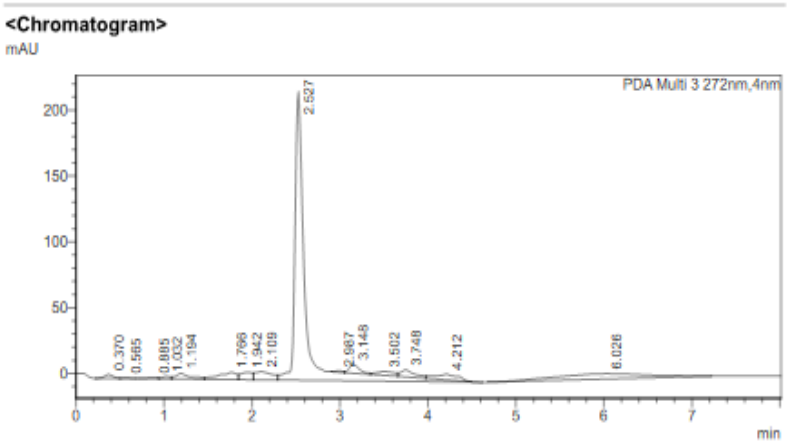
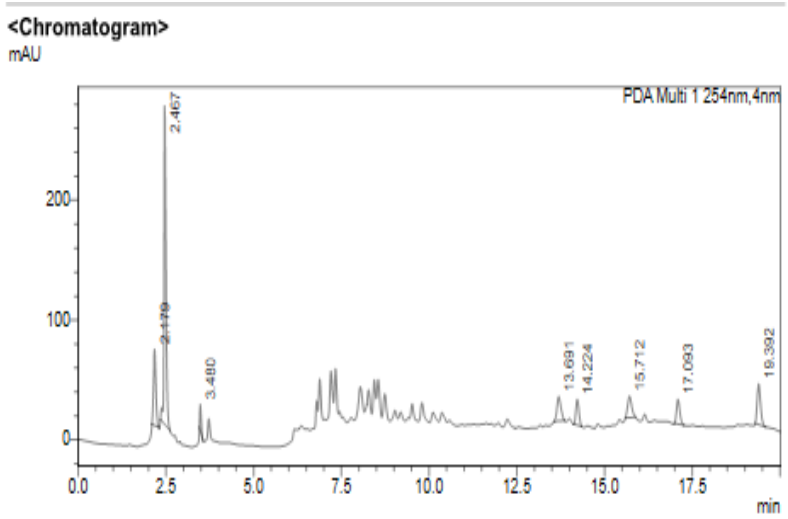


Figure 1: Chromatogram of Gallic acid standard



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On X-axis = Retention time,
On Y-axis = Measure of intensity of absorbance
Figure 2: Chromatogram of the sample (fenugreek leaves)

3.3 DPPH radical scavenging assay

After performing the DPPH assay for the anti-oxidant activity, it was found that there was a decrease in absorbance of DPPH radical caused byantioxidants, because of the reaction betweenantioxidant molecule and radical progresses, whichresults in the scavenging of the radical by hydrogendonating. The illustrations were made that there was a significant decrease in the concentration of DPPH due to scavengingactivity of fenugreek extract.

4. DISCUSSION

Nature has been a source of medicinal agent for thousands of years and an impressive number of modern drugs have been isolated from natural sources [28]. Plants have the ability to produce a large variety of secondary metabolites such as saponins, tannins, phenols, alkaloids, triterpens and phytosterols. In present qualitative analysis of fenugreek leaves extract, the result of the preliminary phytochemical screening was carried out on the methanolic extracts of the sample and revealed the presence of a wide range of phytoconstituents including flavonoids, tannins, phenols, and terpenoids, supporting the reason for its wide range of biological activities as shown in Table 1. Phenols possess biological properties such as anti-apoptosis, anti-aging, anti-carcinogen,anti-inflammation, anti-atherosclerosis,cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities [29].

However for the quantitative analysis, fenugreek leaf extracts was examined for its phenolics composition using HPLC. Data from HPLC analysis revealed that the quantity of phenols, evidently, gallic acid was present in high amount (544mg/g) in the leaf extracts, on taking gallic acid as a standard. Phenols and flavonoids are very important plant constituents because of their anti-oxidant activity[30]. The anti-oxidant activity of phenolic compounds is mainly due to their redox properties which play an important role as free radical scavengers, reducing agents, quenchers of singlet oxygen and complexes of pro-oxidant metals [31]. The result obtained from the quantitative analysis of fenugreek leaves extract showed that alcohol is a better solvent for the extraction of phenols since it gave a very high yield of gallic acid. This is in agreement with the study by Tsao and Deng *(2004)* [32] which showed that phenolic acids are generally better extracted using alcohols, water or a mixture of water and alcohols. *Bukhari et al. (2008)* [33] had shown higher phenolic compounds in ethanol extract of fenugreek seed. Similar results were observed in the present study with fenugreek leaves.

The antioxidant capacities of the fenugreek extracts were analyzed by using the free radical scavenging capacity (DPPH). The DPPH test is the oldest indirect method for determining the antioxidant activity which is based on the ability of the stable free radical 2, 2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenols [34]. The methanol extract recorded the highest phenol content and also had the highest scavenging activity. There was a linear correlation between the anti-oxidant activity and total phenol content of fenugreek leaves. This suggests that the phenolic compounds contributed significantly to the anti-oxidant capacity of the investigated plant species. The result was

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consistent with the findings of *Tawaha et al. (2007)* [35] who reported positive correlation between total phenols and scavenging activity.

5. CONCLUSION

From the present work, it could be concluded that fenugreek leaves extract contain various important secondary metabolites, of which phenolic compound like gallic acid is present in high amount and the solvent play a vital role in the extraction of the constituents. As methanol is highly polar among the solvents, therefore it contains a high yieldof phenolic as compared to the other solvents. A methanolic extract of fenugreek leaves was examined for its antioxidant activity. The antioxidant activity could be correlated with the polyphenolic components present inthe extract. The results gained by these methods providesome important factors responsible for the antioxidantpotential of fenugreek leaves.

The anti-oxidant capacity of the plant extract is mainly reliant on the phenolic compound. One of the important secondary metabolite includes the phenolic compound, which are synthesized by the plants as an adaptation, in response to the biotic and abiotic stress. However, the results were concluded by the quantitative analysis, in accordance to the standard method. Thus, it demonstrates the total gallic acid content present in the fenugreek extract.

Due to the presence of gallic acid in the plant extract, fenugreek shows good anti-oxidant, anti-septic, anti-cancerous properties and is useful in preventing diseases like cancers, diabetes, stroke, and atherosclerosis.Since DPPH molecule contains stable free radical, it is extensively used to assess the radical scavenging ability of anti-oxidants.

CONFLICT OF INTEREST

None

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QUALITATIVE STUDIES ON COLOUR REMOVAL FROM DISTILLERY EFFLUENT USING LOW COST ADSORBENT MATERIALS UNDER STATIC BATCH MODE

Ishwar Chandra[1,2,*], N Ramesh[2] , A Upadhyay[1]
[1]M Visvesvaraya Institute of Technology, Krishnadeveraya Nagar, Hunasamaranahalli Bangalore-562157,
[2]Department of Biotechnology, Ph.D scholar at Reva university, Bangalore.
[3]Dean, Faculty of Science and Technology & Training, Placement &Planning, REVA University, Bangalore.

ABSTRACT: Distilleries use molasses as feedstock for ethanol production and generate large volume of dark brown coloured effluent known as spent wash.Colour in spent wash is due to the presence of melanoidins which contributes to high COD. Untreated spent wash is capable of polluting water & land. Preliminary studies on colour and odour removal from distillery spent wash were performed using low cost adsorbent materials. The objective of this work is to examine 5 carrier materials (CM) *viz.* sand, soil, bagasse, flyash (FA), and distillery yeast sludge (DYS) for color removal from biomethanated distillery spent wash under static -batch mode. Physiochemical & biological characterization of all the carrier materials and spent wash was performed. The percentage decolourisation with soil was more than 60%, with bagasse more than 70% and with sand more than 60% was achieved, while distillery yeast sludge and FA showed less than 60% decolourisation. There was no significant effect of pH on the decolourisation process. All the CM were able to remove odour from spent wash except DYS.

KEYWORDS: Colour removal, distillery spent wash, Static condition, low cost adsorbent.

***Corresponding Author:** Ishwar Chandra.
M Visvesvaraya Institute of Technology, Krishnadeveraya Nagar, Hunasamaranahalli Bangalore-562157

1. INTRODUCTION

Molasses based distilleries are attracting considerable interest due to their hazardous waste water released in huge volume. They are featured under the “Red Category” list as per the India Ministry of Environment and Forest (MoEF)[1]. This waste water is called as spent wash and it is capable of polluting recipient water by causing eutrophication. They also pollute agricultural land by altering their chemical and biological properties [2-8]. The characteristic feature of spent wash are, low pH (3.5- 5.0), high volume of 8-15 liter for each liter of ethanol [9], dark brown colour, high chemical oxygen demand (COD) [10], offensive odour and recalcitrant nature [10-11].

As per latest reports, there are around 397 distilleries operating in India producing more than 40 billion liter of spent wash annually, which is partially treated before its discharge, leading to scarcity of fresh water and increase in waste water [12-13]. By 2020 distillery will have to probably shut down the production of ethanol, if availability of fresh process water is not there. Treatment of this voluminous effluent is either done by physiochemical or biological methods. Physiochemical methods are highly effective in reducing COD, but methods like adsorption using activated carbon [14], flocculation & coagulation [15] generate secondary pollutants. Advanced methods like incineration in multiple effect evaporator suggested by AIDA & CPCB, India, advanced oxidation techniques, *viz.* Ultrasound and Ozone [16] ultrafilteration & reverse osmosis [17], and membrane technology [18] are not cost effective and difficult to scale-up. On the other hand,

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biological treatment involves sequential anaerobic digestion followed by aerobic treatment. This approach recovers methane gas and leaves behind biomethanated distillery spent wash (BDSW), which is later treated in aerated lagoons [19]. Biological treatment is highly inefficient over chemical methods. A major difficulty in treatment of spent wash is the presence of a polymer and a natural browning pigment called melanoidins [20-21]. The melanoidins have empirical formula of $C_{17\text{-}18}H_{26\text{-}27}O_{10}N$ with molecular weight of 5-40 KDa [22]. Melanoidinfrom BDSW have diverse structure & elemental composition [23-24], antioxidant nature [25], antimicrobial property [26]& toxic to the microbes used in spent wash treatment. Chiefly maillard product of sucrose–aspartic acid (SAA-MP) is the major colour imparting melanoidins in BDSW [27-28].

CPCB India have instructed distilleries to opt for zero-discharge by utilizing the diluted spent wash for ancillary purpose, gardening and blending with concrete for construction etc. Many authors have reported controlled use of BDSW for fertigation in top vegetables (Creepers), groundnuts, sugarcane cultivation, since its a rich source of nutrients [29-30]. Literature also reports the use of low cost adsorbent material to remove melanoidins from BDSW. The aim of this paper is to present low cost readily available adsorbents for decolourisation of BDSW under static batch mode.

2. MATERIALS AND METHODS

2.1 Characterisation of Spent wash and carrier materials

The spent wash used for the study was collected from a distillery unit in Karnataka, India. The sample was diluted to desired dilution using deionized water to get desired strength (COD). Physicochemical & biological parameters like colour, COD, total dissolved solid (TDS), microbial count in BDSW were estimated as per standard methods. BDSW was characterized for Colour using UV visible double beam spectrophotometer and later calibrated against COD. All the carrier materials were characterised for size using sieve analysis equipment of known cut-off sizes.

2.2 Decolourisation studies

Soil, sand, bagasse, DYS and fly ash were used as carrier material/adsorbent for the study; all the adsorbents were obtained from the near vicinity of the distillery plant. The adsorbents were sieved by using a sieve set and then used for the study. Batch adsorption studies were performed with 5 ml of carrier material dispensed in 40 ml of BDSW of different strength. All the samples are incubated at constant temperature of 35 ± 2^0C under static conditions. The analysis of sample was done after 17 days, the samples were centrifuged using cooling centrifugation (4^0C) at 8,000 rpm for 10 minutes to separate the carrier material from BDSW. The supernatant was read for OD_{475}using a double beam UV–vis spectrophotometer and percentage of colour removal was calculated as the decrease in colour intensity of the decolourised sample against that of initial colour of the original BDSW:

$$\text{Percent colour removal} = \frac{Initial\ OD - Final\ OD}{Initial\ OD} \times 100$$

The entire assay were performed in triplicates, the results interpreted are averages and compared with control. To study the effect of pH on decolourisation process, the sample was adjusted to pH 7 using 0.1 M H_2SO_4 or 0.1 M NaOH. BDSW of pH 7 was subjected to decolourisation with carrier materials. After 17 days of incubation, percentage of decolourisation was calculated.

3. RESULTS AND DISCUSSION

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3.1 Characteristics of BDSW and Carrier Material

Anaerobically digested distillery spent wash collected after biomethanation process was dark brown in colour. BDSW showed high COD (by open reflux method) of 22,000mg/l (σ=50mg/l) and TDS of 0.225g/ml. The microbial count in BDSW showed a total of 32×10^8 CFU/ml. Various dilutions of BDSW exhibited perfect positive correlation with COD, which means colour of the spent wash is responsible for high COD. The volume surface mean Dia of soil, sand, bagasse, DYS, fly ash is 0.184524, 0.200155, 0.200152, 0.174, FA-0.225 mm respectively. The microbial count in soil, sand, bagasse, DYS, fly ash is 51×10^6 CFU /g, 238×10^6 CFU /g, 63×10^3 CFU /g, 20×10^3 CFU /g, 15×10^3 CFU /g.

3.2 Decolourisation studies

Soil, sand, and fly ash showed the maximum decolourisation at low strength BDSW *i.e* 10 %.v/v. Treatment of BDSW with soil resulted in highest decolourisation of 73.33%. Sand showed maximum decolourisation of 66.086% while fly ash had maximum decolourisation of 65.217%. On increasing the strength of BDSW there was a decrease in percent decolourisation.

Studies with DYS indicated initial increase in percent decolourisation followed by a decline phase of percentage decolourisation on increasing strength of BDSW. DYS was capable of reducing the colour to the tune of 62.958% for 20% (v/v) BDSW.

Studies with bagasse showed a maximum decolourisation of 59.646% for 80% BDSW. At low strength BDSW the percentage decolourisation was low, but with increase in strength of BDSW the adsoption of melanoidin also increased significantly.

All the carrier materials were also able to remove the odour except DYS. Baggase gave a sweet smell to the BDSW after treatment.

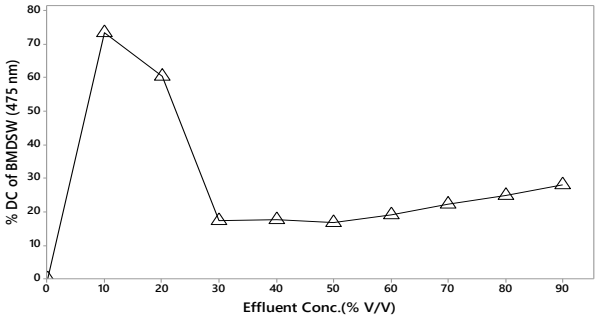

Figure 1: Percent Decolourisation (%DC) using soil under static mode

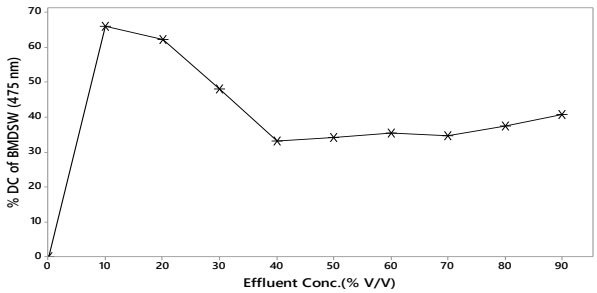

Figure 2: Percent Decolourisation (%DC) using sand under static mode

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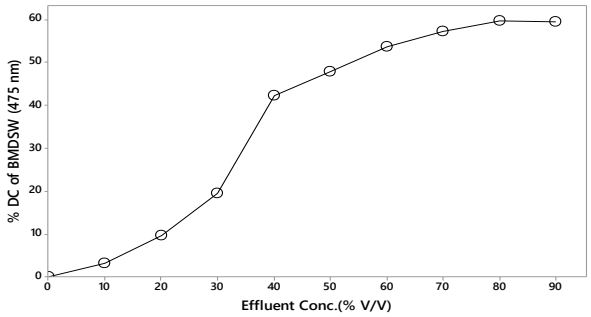

Figure 3: Percent Decolourisation (%DC) using bagasse under static mode

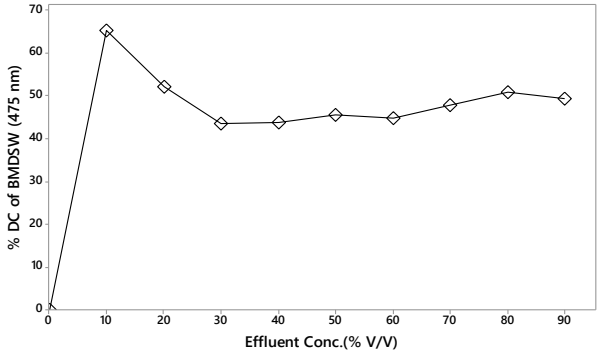

Figure 4: Percent Decolourisation (%DC) using fly ash under static mode

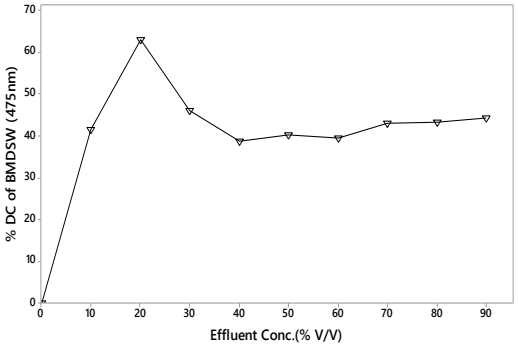

Figure 5: Percent Decolourisation (%DC) using DYS under static mode

While at corrected pH 7, highest decolourisation was shown by Bagasse (63.02547771) followed by Soil (55.873016) and last by Sand (43.74233129). pH does not have any significant effect on the colour adsorption, because decolourisation at pH 7 is lesser when compared to decolourisation at regular pH.

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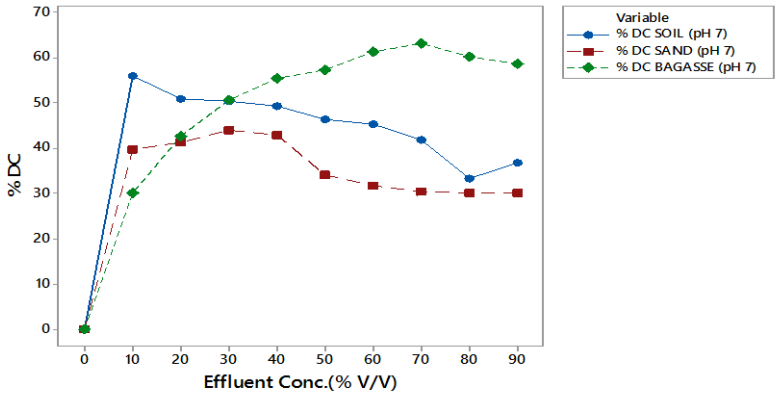

Figure 6: Percent Decolourisation (%DC) under static mode at corrected pH of 7

4. CONCLUSION

Molasses spent wash from alcohol distillery contains melanoidin, a dark brown recalcitrant pigment. It is not easily biodegraded/adsorbed due to complex nature and causes a number of problems leading to environmental damage. Batch adsorption studies under static mode shows that, soil, sand and bagasse could be excellent carrier materials because they can remove considerable amount of colour from the spent wash. All the adsorbents are cost effective, readily available and easy to dispose, which will not lead to secondary pollution. This work could be a preliminary study and If these materials are combined in an appropriate formulation and used as a static packed bed will probably lead to decolourisation of spent wash by a good amount.

CONFLICT OF INTEREST

None

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DETERMINATION OF ANTI-BACTERIAL ACTIVITY OF *LEUCUS ASPERA* PLANT

Rakshit Jujare[1],Rishab Aditya[1], Rakesh Sarkar[1], Jayashree S[1]*
[1]Department of Biochemistry, REVA University, Bengaluru - 560064

ABSTRACT:
Leucus aspera is a plant of the family Labiatiae, is a common aromatic herb found in temperate regions of America, Asia etc. In this study, we are concerned with evaluating the antibacterial activity of *L. aspera*. The plant parts were sun dried, grinded, and then dissolved in methanol and soaked for 4 days. The dried plant parts were removed and the filtrate was further concentrated using rotary evaporator. The filtrate was then again re-dissolved in 80% methanol to obtain 2mg/mL of extract. The bacteria species chosen were those of *S. aureus* and *E. coli* with Penicillin as the standard antibiotic. Well diffusion method was performed and results were obtained in the form of zone of inhibition.The zone of inhibition for *E. coli* was obtained to be higher than *S. aureus* while in general, the zone of inhibition for the plant was found to be lower compared to the antibiotic, thus showing its potent antibacterial activity, which is mainly due to the presence of tannins, glycosides, and flavonoids in them, which are all secondary metabolites. Due to its significant antibacterial activity, this plant can be used in formulations of medicines related to anti-rheumatic, anti-inflammatory and also in the production of new antibiotics.

KEYWORDS: *Leucas aspera*, *S. aureus, E. coli* , antibacterial

***Corresponding author**: Dr. Jayashree S, HOD Department of Biochemistry, REVA University, Bengaluru – 560064. Email: jayashrees@reva.edu.in

1. INTRODUCTION

In recent years, there has been an increasing interest in the study of medicinal plants and their medicinal value in different parts of the world. These medicinal plants are found to possess potent pharmacological activities, low toxicity and economic viability [1]. The different compounds extracted from the medicinal plants are found to possess a wide range of activities including, antibacterial, and anti - inflammatory and also anti-cancerous properties. They are found to provide cure against ageing, neurodegenerative and atherosclerosis. Multi – drug resistance by pathogens to conventionally used antibiotics led to the need for identifying novel anti infectives with mechanisms of actions that cannot be circumvented by the microbes [2].

L. aspera, belonging to the family of Labiatae is a common herb, found as weed in Asia, Africa and other tropical countries. The whole plant is taken orally as anti-inflammatory, analgesic and antipyretic properties. The entire plant is used as an insecticide and indicated in traditional medicine for cough, colds etc. Compounds isolated from the plant include long chain aliphatic and aromatic compounds, triterpenes, sterols, and novel phenolic compounds, flavonnoids. These compounds are synthesized by primary or rather secondary metabolism of living organisms [3] Apart from this, the plant also possesses wound healing property.In this study, we have studied the antibacterial activity of *L. aspera* extracts with *S. aureus* and *E. coli* bacteria species. The roots, leaves and flowers are mainly used in treating blood pressure, cold, whooping cough, asthma and stomach ache [4].

While chemical investigation of the volatile oil of the African *L. aspera* detected carvone, carvacrol, and methanol as major components, a recent report by Joshi et.al (2016) on the volatile oil of the Indian *L. aspera* uncovered that the oil is rich in sesquiterpene

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hydrocarbons (47.7 %), where ß – caryophyllene (51.1 %) was the fundamental constituent with a percentage of 34.2% [5]. The first report on the volatile constituents of L. aspera indicates the predominance of sesquiterpenes (36.9%) over monoterpenes (26.4%) in the leaf volatiles [6].

2. MATERIALS AND METHODS

2.1 Plant material The plant material was collected from the locality of Kattigenahalli, Yelahanka. The aerial parts of the plant , viz., leaves , stem, flowers and petals were collected and trimmed [7]. Then the segregated parts were cut into small size and then sun dried for a week [8].

2.2 Extract preparation All the parts of the plant (root, leaf, flower, and stem) were grounded into powdered form with a grinder together and it was collected to be 20 gm. Then, the plant parts were soaked in80% methanol in a beaker and left to soak for 4 days at room temperature. Then, the dry plant parts were removed by filtration using cheese cloth .The extract was concentrated to dryness under reduced pressure and controlled temperature (40-50^{0}C) using a rotoevaporator [9]. The dried plant extract was then re-dissolved in 80% methanol in order to obtain a solution whose concentration will be 2mg/ml of extract which were then used for further assay of antibacterial activity.

2.3 Microorganisms Gram positive bacteria *S. aureus* and Gram negative organism *Escherechia coli* were chosen as model organisms to test for antimicrobial activity as they are the most dominant and disease causing species of bacteria. *S. aureus* is known to cause a wide range of disorders ranging from skin infections such as impetigo, pimples, boils, to life – threatening diseases such as pneumonia, meningitis, osteomyelitis

Escherechia coli is a Gram negative bacteria, that is normally found in the gut of animals, and causes a wide variety of infections including that of gastrointestinal infections, diarrhoea and food poisoning.

All the stock cultures were collected from the Biotechnology department of REVA University. All the strains were maintained on nutrient agar at 4ºC and were subcultured every month [10]. Confirmation test was carried out to detect whether the stock culture of *S. aureus* was of that bacteria only or of a different one (Mannitol salt agar was used in the detection of *S. aureus* with *S. aureus* giving yellow colonies). This is because Mannitol salt agar is a differential media. The clinical isolates were biochemically and serologically characterized by standard methods [11]

2.4 Antibacterial activity The antibacterial activity of the plant was performed by agar diffusion method [12]. The agar media used for this antibacterial activity is Mueller Hinton Agar media, which is the most commonly used agar media for antimicrobial susceptibility testing. All of the bacterial strains were grown and maintained on Mueller Hinton agar media at 37° C and pH (7.3^{+}- 0.2) [13]. The composition of this media is listed in table 1.

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Table 1. Component of Preparation of Mueller – Hinton agar media

INGREDIENTS	GRAM/LTR
Beef extract	0.2
Acid hydrolysate of casein	1.75
Starch	0.15
Agar	1.7
Distilled water	100 ml

a) Suspend 3.8 gm of medium in 100 mL of distilled water
b) Heat with frequent agitation and boil for one minute to completely dissolve the media.
c) Autoclave the media at 121 degree centigrade for 15 minutes. Cool to room temperature.
d) Pour cooled Mueller – Hinton agar into sterile petridish on a level, horizontal surface to give uniform depth
e) Allow the medium to cool to room temperature
f) Check the medium pH to be finalised to 7.0-7.3 at 25 degree centigrade.

For each of the test organisms, the pre-cultures were taken from stock cultures and were grown in nutrient broth at 37ºC for 24 h [14].The test microbes were taken from the broth with an inoculating loop and plated in the petriplate containing the agar media. The agar was melted (50ºC) and the microorganism cultures were then added aseptically to the agar medium at 45ºC in plates and poured into sterile petriplate to give a solid plate [15] .The microorganisms were inoculated on Mueller Hinton agar (MHA) and spread uniformly using a sterile spreader [16]. Two petriplates were taken – one petriplate of *E. coli* and another of *S. aureus.* For agar well diffusion method, a well was prepared in the plates with the help of a cork – borer (0.85 cm) [17]. The petriplates were plated with the bacteria before the wells were bore.

In the *E. coli* petriplate, in one well was added the test antibiotic – Penicillin (200 micro litre), whose concentration was 0.1 mg/ml, in one well and in one well the plant extract was added of the same volume.

The same treatment was done for the petriplate with *S. aureus*. The plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37ºC for 24 h [18]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms [19].

Zone of inhibition is defined as the area around the well which shows no growth of microorganisms, as they are inhibited by the antibacterial agent in the extract. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone [20]. All the experiments were performed in triplicate and the results (mm of zone of inhibition) were expressed as mean values [21]. The resulting clear zones were measured by a transparent scale [22].

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3. RESULTS

For testing the anti pathogenic activity of plant extract we performed the well diffusion essay method. Initially we validated the optimum concentration of standard antibiotic (penicillin) to inhibit the growth of both *S. aureus and E. coli.* The standard antibiotic used was penicillin, which showed maximum inhibition of 30±0.5mm for *S. aureus* and 20±0.3mm for *E. coli* respectively. Comparatively the plant extract showed 15±0.03mm and 6±0.2mm inhibition respectively, to *S. aureus* and *E. coli.*

The zone of inhibition of *E. coli* is found to be lesser than that of *S. aureus*, indicating that the plant is more potent against *S. aureus* than *E. coli.*

Table 2. Represents the zone of inhibition of *S. aureus* and *E. coli* by plant extract in respect of standard antbiotics.

	Staphylococcus aureus	***Escherechia coli***
Plant extract (2mg/ml)	15±0.3mm	6±0.2mm
Penicillin (0.1mg/ml)	30±0.5mm	20±0.3mm

4. DISCUSSION

Plants have long been associated to providing cure against a wide range of disorders and harmful pathogens. In today's medical world, a wide range of drugs are made from plants. The use of some antibiotics is no longer recommended because of the potency of widespread resistance to them [23]. Thus it was necessary to evaluate the anti microbial activity of the plant *L. aspera.*

In this study, we have evaluated the antibacterial activity of the methanolic extract of the entire plant by agar diffusion method. The microorganisms chosen for this study was – *E. coli and S. aureus* as these are very harmful disease causing pathogens, which cause a wide range of disorders. Methicillin resistant *S. aureus* (MRSA) is an increasing problem in human medicine, which may have catastrophic effects [24]. Previous research studies with other medicinal plants such as Tulsi have also revealed the significant antibacterial activity of its oil. Tulsi oil at concentrations of 4.5 and 2.25% completely inhibited the growth of *Staphylococcus aureus*, including MRSA and *E. coli*, while it partly inhibited the growth of *P. aeruginosa* [25].

The zone of inhibition for the antibiotic is usually found to be higher than that of the methanolic extract of the plant as because the plant extract is in crude form and in crude form, the plant has lower concentrations of the secondary metabolites and bioactive compounds. However, the study revealed that the plant is more potent against S*taphylococcus aureus* than *Escherechia coli.* Gram - positive strains were found to be more sensitive than Gram – negative organisms to the extract on average [26].

However different zone of inhibition may be obtained in case of different solvents. The ethanolic extract of the whole plant exhibited potent bactericidal activity at higher concentrations in a time and dose dependent manner against *E. Coli* in colony forming unit method [27]. However the methanolic extract is considered to be more effective in controlling the growth of pathogenic strains to a great extent. Only the methanol extract is solely responsible for antibacterial and antifungal activity and can be used as a broad -

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spectrum antimicrobial agent [28] . Thus it can be said that the plant can be used in pharmaceuticals as their extracts may yield significant results against certain bacteria and hence, this plant can be a boon to the human race especially after the multi drug resistance of bacteria prevalent in today's world.

5. CONCLUSION

In conclusion, the study reveals that the plant shows greater zone of inhibition for *S. aureus* than *E. coli* and the zone of inhibition of the plant extract is lesser compared to the antibiotic as the plant extract is obtained in crude form. Phytochemical analysis needs to be done in order to isolate and characterise the compounds which are responsible for conferring them the antimicrobial property. Thus it is a useful agent for human health and future studies might reveal more properties of the plant.

CONFLICT OF INTEREST

None

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Rukmini Knowledge Park,
Kattigenahalli, Yelahanka, Bengaluru - 560064

+91 80 6622 6622, +91 95388 74444/45