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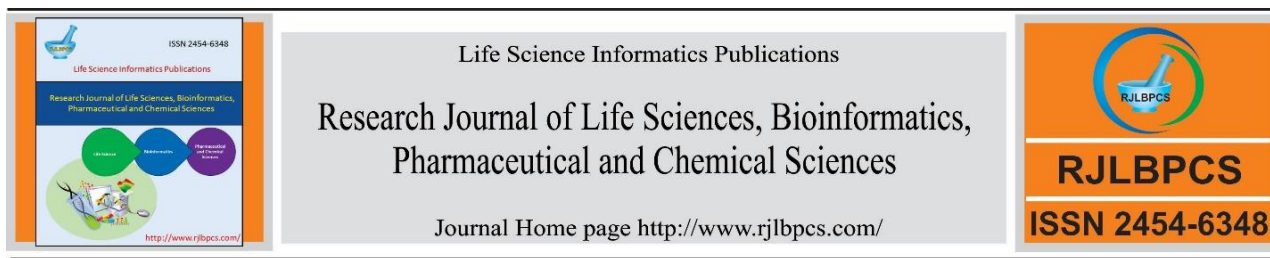


PROF. DEBJANI DASGUPTA

CONVENER, NCIFEH-2018

DIRECTOR, SCHOOL OF BIOTECHNOLOGY AND BIOINFORMATICS

School of Biotechnology and Bioinformatics, D Y Patil Deemed to be University,
Plot no.50, Sector 15, CBD Belapur, Navi Mumbai 400614, INDIA.



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***V.vinefera*: A Promising Source of Resveratrol for Nutraceutical Applications**

A. V. HANDORE¹, S. R. KHANDELWAL^{1*}, A. D. BHOLAY²

¹P.G. Department of Microbiology, HPT Arts and RYK Science College, Nashik -422005
MS, India.

²K.T.H.M. College, Nashik, India.

ABSTRACT: Globally, there is high demand of nutraceuticals for prevention and treatment of health issues since last few decades, consumers and nutritionists are continuously searching better nutraceuticals from natural sources for reducing the negative health impact. Resveratrol, the polyphenolic compound is clinically proved nutraceutical. This compound does not enjoy a wide distribution in the plant world, and has been reported in few fruits and plants employed for human consumption. However, *Vitis vinifera* (Grape plant) is a traditionally accepted dietary source of various phytonutrients. It is an extensively used ancient fruit crop linked with human history during the evolutionary development of humankind. This plant has been extensively used in different *Ayurvedikrasayana*, drugs and formulations like *Drakshasava*, *Draksharishta*, raisin etc. According to the scientific evidences, Resveratrol is accumulated in different parts of this plant. Therefore, present research has been focused on determination of nutraceutical potential of different parts of this traditional plant. Organic extract of different part like leaf lamina, stem, petiole and berry skin of indigenous black cultivar of *V. vinefera* was prepared using 90% Methanol. Total polyphenolic content was studied by Folin-Ciocalteu method. Antioxidant potential was estimated by DPPH assay followed by preliminary detection of resveratrol using the chromogenic test. Quantification of resveratrol in potent sample was carried out by HPLC analysis. Antibacterial activity of the potent sample was carried out against food pathogenic bacteria viz. *S. enterica*, *S. aureus* and *P. aeruginosa* and *E. coli*. The results of Total phenolic content indicated that although, all the parts showed noteworthy amount of total phenolics skin sample showed highest polyphenolic content i.e. 1.17 ± 0.01 mg GAE/g. The order of radical scavenging activity i.e. antioxidant potential was found as, Skin >Petiole>Stem>Leaf lamina. Preliminary screening exhibited comparatively high abundance of polyphenolic resveratrol in skin sample. Concentration of resveratrol in the skin was found to be $10.79 \mu\text{g/gm}$ by HPLC analysis. The maximum antibacterial activity was found against the *S. aureus* whereas, minimum activity was found against *E.coli*. Anti-biofilm approach can provides an alternative to an antibiotic strategy and can help to check the nutraceutical efficacy of sample and the antioxidants will help to avoid various chronic diseases and disorders. Therefore, it can be concluded that as compare to the available sources in market, *V. vinefera* can be used as safe and promising dietary source to formulate nutraceuticals.

KEYWORDS: *V. vinefera*, Polyphenol, Resveratrol, Antioxidants, Nutraceuticals, Food pathogen

***Corresponding Author: Dr. S. R. Khandelwal**

P.G. Department of Microbiology, HPT Arts and RYK Science College, Nashik -422005, MS, India.

Email Address: Sharad_khandelwal13@yahoo.com

1. INTRODUCTION

Nutraceutical is a term devised to describe substances which are not traditionally recognized nutrients but can provide medical or health benefits, including the prevention and treatment of disease. They may range from isolated phyto-nutrients, dietary supplements and specific diets to genetically engineered designer foods and herbal products [1-3]. Globally, there is high demand of nutraceuticals for prevention and treatment of health issues. Since last few decades, consumers and nutritionists are continuously searching for the better nutraceuticals from natural sources in order to reduce the negative effect on human health. It is clinically proved that Resveratrol, the polyphenolic compound can be effectively used as nutraceutical due to its great antioxidant potential. Several scientific research has explained the high appeal of resveratrol as, it can be used against various non- communicable diseases and disorders like cardiovascular diseases, cancer, neurodegenerative disease and diabetes etc. This compound does not enjoy a wide distribution in the plant world, and has been reported in few fruits and vegetables employed for human consumption. However, *Vitisvinifera* (Grape plant), the basket of various bioactive compounds is an extensively used ancient fruit crop linked with human history during the evolutionary development of humankind. This plant has been extensively used in different *Ayurvedikrasayana*, drugs and formulations like *Drakshasava*, *Draksharishta*, raisin etc. According to the scientific evidences, Resveratrol is accumulated in various parts of this plant like leaf, stem, petiole, root, fruit berry, seeds and skin etc. Therefore, present research has been focused on determination of nutraceutical potential of this traditional plant for therapeutic applications.

2. MATERIALS AND METHODS

2.1.Sample Collection

Fresh and healthy aerial plant parts viz. leaf lamina, stem and petiole and skin of healthy berries of most commonly grown table grape cultivar of *V.vinifera* (Sharad seedless-black) was randomly collected from vineyard of Nashik valley, Maharashtra India during June 2016.

2.2.Extraction

Collected plant material was carefully separated, cleaned, shade dried for fifteen days at room temperature and mechanically grinded to fine powder. The powder (10%) was subjected to solvent extraction with 90% methanol. It was allowed to incubate at room temperature with gentle shaking for 72 hours. Supernatants were further evaporated to dryness. Hygroscopic yield of extract was noted with respect to dried plant material powder. The extracts thus obtained were dissolved in methanol at the concentration of 1 mg/1 ml for further analysis.

2.3.Determination of Total Phenolic Content

Total phenolic content was determined by Folin- Ciocalteau method of Zheng and Wang. Absorbance of the test samples were measured at 725 nm and content of phenolic in extracts were calculated using a Gallic acid (0.1-1.0 mg mL⁻¹) standard curve. The results were expressed in terms of gallic acid equivalent (mg of GAE/g of extract) All the tests were carried out in triplicate

[4-5].

2.4.DPPH Radical-Scavenging Activity

The antioxidant potential of test samples were estimated by the free radical scavenging ability with reference to the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) as per spectrophotometric method reported by S. Sannigrahi, 2010 with few modifications [6]. Briefly, plant extract (100µl) was added into 100 µL methanolic solution of DPPH (0.1mM) final volume was made up to 2 mL with methanol. Whole mixture was shaken vigorously and incubated (30 min) in dark at room temperature. Absorbance was measured at 517 nm. Radical-Scavenging Activity toward DPPH was estimated from the equation as: **% Inhibition = [A control –A sample / A control] x 100**

2.5.Preliminary Screening of Resveratrol

The test samples were subjected to chromogenic reaction for preliminary detection of polyphenolicresveratrol .Chromogenic agent was prepared by mixing, 0.1 % FeCl₃: 0.1 % K₃ [Fe (CN)₆] = 1:1 (v/v). 2 mL test sample was added to 2ml methanol and 2-3 drops of chromogenic agent. Blue colour with greenish tinge indicates presence of polyphenolic compound like resveratrol [7].

2.6.HPLC Analysis

Determination of Trans- resveratrol was carried out by using HPLC (Analytical Technologies Ltd.- HPLC 3000 series gradient binary system).The system was equipped with UV detector (UV-3000M) .Column configurations were Grace, RP-C18, 250 mm× 4.6 mm (ID), Particle size 5 µm held at room temperature using the solvent system, Methanol: Water (80:20) (v/v).Standard Trans resveratrol (25 ppm) was used as reference. Each liquid sample was filtered through a 0.22 micron cellulose membrane filter, Sample detection was carried out at 306 nm with 20µL of sample injection at 0.8 ml/min (Isocratically). Identification and quantification of Trans resveratrol was carried out by comparing the retention times and the peak areas with standard resveratrol.

2.7.Antimicrobial Activity Tests

Standard Bacterial cultures of Gram negative bacteria viz. *E.coli* (NCIM-2931), *Salmonella enterica* (NCIM -5256), *Pseudomonas aeruginosa* (NCIM -5210) and Gram positive bacteria *S.aureus* (NCIM-2127), were obtained from the National Collection of Industrial Microorganisms (NCIM, National Chemical Laboratory, and Pune, India). All the microorganisms were maintained on nutrient agar slants at 4°C.Standard cultures were individually inoculated in Nutrient broth (Hi-Media) to achieve a specified inoculums size. The antimicrobial effect of the potent sample was tested using the agar well diffusion method. Approximately 15 mL of Nutrient agar was seeded with 0.1 ml of overnight grown bacterial cultures. Each agar well was loaded with 10µL samples. After pre-diffusion, plates were incubated at 37°C for 24 hours and observed for zone of inhibition. All the tests were carried out in duplicate [8].

3. RESULTS AND DISCUSSION

3.1.Determination of Total Phenolic Content

It was observed that the skin extract showed deep Blue colour with greenish tinge as compare to the samples of petiole stem and leaf lamina .The results showed that the intensity of the reaction of phenolic compounds with Folin–Ciocalteu reagent follows the classical principle of structure-activity relationship, in which the order of activity is proportional to the availability of hydroxyl groups present on the aromatic ring and influenced by groups that can decrease or increase the reductive potential of the molecule [9-10]. It was revealed that the skin extract showed highest phenolic content (1.17 ± 0.01) whereas, least phenolic content was shown by stem (0.56 ± 0.05 mg/g GAE). Therefore, the results indicated that although all the parts showed noteworthy amount of total phenolics, berry skin is rich source of polyphenolic compounds [Table.1].

Test samples	Total Phenolic Content (mg GAE/g)Mean $\pm$ SD
Leaf Lamina	0.59 ± 0.05
Stem	0.56 ± 0.05
Petiole	1.13 ± 0.29
Skin	1.17 ± 0.01

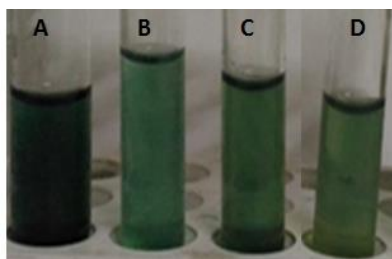
Table 1: Total Phenolic Content of different of parts of Sharad Seedless cultivar

3.2.Antioxidant Evaluation

The DPPH radical scavenging activity of different aerial parts of *V.vinifera* was carried out in triplicates. Ascorbic acid was used as reference compound. It was observed that Skin extract showed highest radical scavenging activity i.e. % inhibition (85.32%) as compare to petiole (82.22%), stem (70.23 %) and leaf lamina (46.34%). The order of radical scavenging activity extracts was found as, Skin > Petiole > Stem > Leaf lamina.

3.3.Preliminary Screening of Resveratrol

Preliminary screening of the test samples by using chromogenic reaction showed blue colouration with greenish tinge indicated presence of polyphenolic compound like resveratrol. It was found that the skin extract showed dark colouration indicating comparatively high abundance of resveratrol than the other parts under study. Therefore, it was used as a potent sample for further study.



A. Skin B. Leaf Lamina C. Stem D. Petiole

Figure 1: Chromogenic test by extracts of *V.vinefera*

3.4.HPLC Analysis

During the HPLC analysis, quantification of resveratrol in potent sample was carried out by comparing the retention time of sample with standard resveratrol ($R_t = 3.6$). The concentration of resveratrol in the skin extract was found to be $10.79 \mu\text{g/gm}$. [Fig.2]

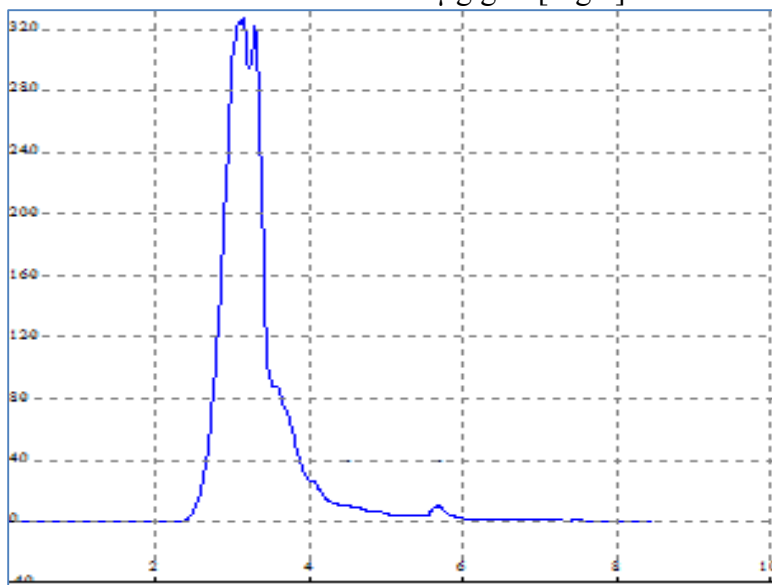
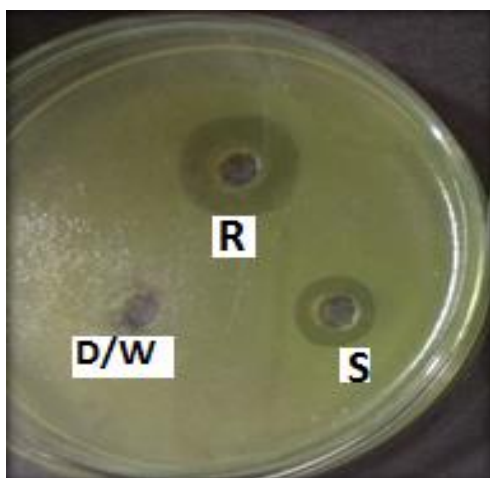


Figure 2: HPLC chromatogram of potent sample

3.5.Antibacterial Activity

It is reported that biofilm formation is associated with the bacterial infection and mechanism of antimicrobial resistance. Thus, anti-biofilm approach can not only provides an alternative to an antibiotic strategy [11] but also can be helpful for determination of nutraceutical efficacy of sample. Therefore, the potent sample showing comparatively high resveratrol content was subjected to antibacterial activity against some common food pathogenic bacteria.



[R: Standard Resveratrol, S: Potent Sample]

Figure 3: Antibacterial activity of skin extract against *S.aureus*

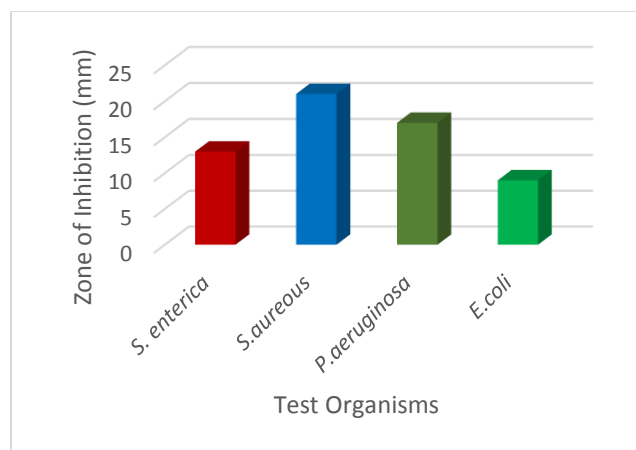


Figure 4: Zone of inhibition of potent sample against test organisms

During antimicrobial study, it was found that the sample showed noteworthy antibacterial activity against food pathogenic bacteria like *S. enterica*, *S. aureus* and *P. aeruginosa*. whereas, comparatively less inhibitory activity was shown by *E. coli*. The inhibition zones by resveratrol rich sample were ranging from 9 to 21 mm. It was observed that highest zone of inhibition was found against *S. aureus* [Fig.3]. Whereas less zone of inhibition was observed against *E. coli*. [Fig.4]. The difference in susceptibilities toward resveratrol containing sample might be attributed due to the presence of a hydrophilic outer membrane in Gram-negative bacteria which is absent in Gram-positive bacteria [12]. It is reported that the possible antibacterial mechanism of action of resveratrol includes DNA damage, cell division impairment, oxidative membrane damage, and also metabolic enzymes inhibition. There are some important reports showing the activities of resveratrol in inhibiting biofilm formation and removing the biofilm which has been already formed against various foodborne pathogens. It is reported that resveratrol has good potential to inhibit the biofilm formation by *S. aureus* [13] also various reports are available showing the antibacterial efficacy of resveratrol against *S. enterica* serovar *Typhimurium* as well as *P. aeruginosa*. It is reported that resveratrol did not result in generation of ROS in *E. coli*. However, the study suggested that the inhibition of *E. coli* by resveratrol may be resulted through site-specific oxidative membrane damage [14-17]. According to the findings of R. Paolillo et al (2011), these actions are mediated at micro molar levels compatible with the concentrations of free polyphenolic compound like resveratrol in biological fluids after the ingestion of polyphenol-rich foods and may readily exert a protective effect on ruffled intestinal epithelial cells and reduce inflammation therefore support its nutraceutical potential of any sample [18].

4. CONCLUSION

In the present study, extracts of different parts of *V. vinefera* were screened for their total phenolic content, antioxidant activity, and antibacterial potential for determination of their nutraceutical efficacy. The results of Total phenolic content indicated that although all the parts showed noteworthy amount of total phenolics, berry skin showed highest total phenolic content i.e. 1.17 ± 0.01 mg GAE/g. On basis of antioxidant potential and preliminary screening for resveratrol,

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sample of skin extract was selected as potent sample for further study. During the HPLC analysis, concentration of resveratrol was found to be 10.79 µg/gm. Significant antibacterial activity was also found against the food pathogenic bacteria. The maximum antibacterial activity was found against the *S. aureus* and minimum activity was against *E. coli*. Anti-biofilm approach can provide an alternative to an antibiotic strategy and can help to check the nutraceutical efficacy of sample and the antioxidants will help to avoid various chronic diseases and disorders. Therefore, it can be concluded that as compared to the available sources in market, *V. vinefera* can be used as a safe and promising dietary source to formulate nutraceuticals.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors declare no conflict of interest relevant to this article

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Meta-Analysis of Breast, Cervical, Colorectal and Ovarian Cancers Reveals New Candidates with Potential Role in Regulating Treatment Modalities

H. PATADIA, A. GANGAWANE*

Parul University, Waghodia, Gujarat, India.

ABSTRACT: One of the Holy Grail for cancer researchers worldwide has been the search of better and novel targets to design more efficient targeted therapies. With the advent of genomic technologies it has been found that most of the cancers have oncogene addiction. While targeting the oncogenic pathways, secondary mutations develop in RAS and PI3K pathways [1]. This leads to Acquired Resistance, which necessitates the need of dual target therapy or other forms of combinatorial treatment regimen. We believe that oncogene and tumor suppressor gene mutations serve as cancer drivers and also increase the mutation rates in cancer cells. Furthermore, these mutations may also drive the cellular system for increased flux in metabolism to propel unrestricted cell proliferation. Our project aims at identification of other cellular components that supports oncogene addiction. By linking gene expression changes linked to tumorigenic state, we could identify more than 600 candidates from data of independent screens on public databases such as CGED, dbDEPC 2.0, DriverDB, RASOnD and IntOGen platforms. Gene ontologies of these candidates reveal presence of candidates with novel functionalities and enriched towards specific pathways. Filtering of these candidates based in cancer type and state specific manner has a potential of unveiling many candidates that might have been overlooked or neglected in individual screens. We present an interesting approach to find novel or previously unexplored candidates from many independent and isolated screens and their validation in biopsy samples.

KEYWORDS: Cancer, Meta-analysis, Data mining, Resistance, Transcriptome, Gene ontologies

***Corresponding Author: Dr. Ajit Gangawane**

Parul University, Waghodia, Gujarat, India.

Email Address: ajit.gangawane@paruluniversity.ac.in

1. INTRODUCTION

Over the past two to three decades, the focus on cancer treatments has been changed from chemotherapy to targeted therapeutics. In the process of developing targeted therapeutic approaches, many important genetic interactions have been identified as important contributors to the cancerous phenotypes. Still, finding an ultimate cure for cancer has remained the Holy Grail for researchers. One of the main reasons for this has been the dynamic and rapidly mutating nature of the Cancer cells. One of the ways this was explained better was through the re-categorization of cancerous phenotypes by Hanahan and Weinberg in 2011 [2, 3]. Targeted therapies against each of this category have still not been able to resolve the issue of acquired resistance and hence failure of many clinical drugs. Hence, finding newer targets that has potentially higher therapeutic index and are inevitable for the system is warranted. Cancer cells have an obligatory trait of “oncogene addiction”. For e.g.: the cells develop activating mutation in any components of the EGFR pathway,

the more frequent being in the EGF receptor. Upon treatment, they develop secondary mutations in either downstream component or activate an alternate survival pathway. In many different cancer types such as Melanoma, Lung, Endometrial, Prostate, Colorectal, and other cancers, activating mutations in the EGFR pathway and Insulin signaling pathways are mostly localized on RAS oncogene and PTEN tumor suppressor gene [1, 4]. Constitutive activation of RAS and deletion of PTEN causes enhanced signaling through these pathways in response to targeted therapeutics against either of these pathway components. Much information is available how the EGFR and Insulin signaling pathways are interconnected but their interaction with the other components of the system is unknown.

A concept in genetics called as “Synthetic Lethality” could pave the way to uncover new synthetic interactions of the RAS and other pathways. This phenomenon was discovered in *Drosophila melanogaster* over a century ago but has been widely recognized only in past two decades. Synthetic lethality is a phenomenon in which two independently existing non-lethal mutations are lethal when they coexist in the same cell [5]. With increasing emphasis on the contribution of oncogene addiction in cancer cells, four independent screens were done to find new synthetic interactors of the RAS mutant background using isogenic cells and a panel of different cancer cell lines [6, 7, 8]. Each group proposed different candidates as potential drug target. Such a discrepancy based on the screening platform and the methodology being employed has led to different outcomes. With ongoing efforts in upgradation of technological interventions in increasing the throughput and the sensitivity of the biological research, there is a pile of online data that is derived from screening the expression systems and genomics of the clinical specimen. Collection of tissue banks from biopsy samples have provided accessible source of samples for carrying out high-throughput screens. A recent review has categorically summarized a list of different databases available for many different cancer types [9]. Many a times, the data available on these databases is through literature mining or submission of raw data from independent screens. This bulk of data is not organized and hence, needs a thorough investigation in finding many unexplored candidates warranting further testing. Hence there has been a pressing need of newer screens that can give more relevant outcomes.

This work aims at identifying new genetic interactions in conjunction with oncogenic pathways for particular cancer types through data-mining and filtering from publicly available databases and resources. The data shall be further funneled through various parameters for finding its validity and significance in the study-design directed towards it. Such a data can serve as a source of potential candidates which can be validated through animal models and clinical screenings or cross-referenced against different platforms and resources for its duplicity or recurrence.

2. MATERIALS AND METHODS

2.1.Databases

Due to the usher of lightning fast processing power for computational analysis, a compendium of several databases have been made public for analysis through newer study designs by independent researchers across the world. The different forms of data and the platform on which they are hosted necessitates clear understanding of the type of data to be used for the research purpose. To begin

our work, we have screened several databases before finalizing the type of data to be used for preliminary analysis. The databases that have been screened to be potentially included in the study designs are CGP (Cancer Genome Project), CPTAC (Clinical Proteomic Tumor Analysis Consortium), TCGA (The Cancer Genome Atlas), COSMIC (Catalogue Of Somatic Mutations In Cancer), CancerDR (Cancer Drug Resistance Database), CancerMA (An integrated bioinformatic pipeline for automated microarray data analysis from publicly available databases), CanSAR (Integrated Cancer Drug Discovery Platform), CGED (Cancer Gene Expression Database), dbDEPC3.0 (A database of Differentially Expressed Proteins in Cancer v_3.0), DriverDB (Cancer Driver genes/mutations Database), ccmDB (Cancer Cell Metabolism Database), RasOnD (RAS Oncogene Database) and NCG 6.0 (Network of Cancer Genes). The preliminary search through various platforms made us realize that the presence of comprehensive data on a single platform will serve a good starting point for function as the nucleation of data analysis [10, 11, 12, 13, 14, 15].

2.2.dbDEPC3.0

A database of Differentially Expressed Proteins in Human Cancer was first launched in 2009, updated to version 2.0 in 2011 and further updated to version 3.0 in 2012 which is still in force. This database mines literatures published and its experimental design to provide a comprehensive resource of data generated from different Mass Spectrometry experiments. Over the years, the database has also added higher level annotations by corroborating information about the queried protein from other databases such as COSMIC, dbPTM, DrugBank, KEGG, PhoSigNet, STRING. Currently, the data hosted on this database is collected from 779 different MS experiments for 11,669 unique cancer related differentially expressed proteins across 26 cancer types. It also makes available the data of 213, 0668 different cancer related amino acid variations, 110,343 post translational modifications and 4,908 drugs. Using this resource, data for all different cancers with their sub-types, from cell-line, tissue and fluid sample type covering all 4 study designs were downloaded. This database has also been used for carrying out preliminary enrichment analysis and network studies for the filtered data.

2.3.Functional Annotation and Clustering

For the purpose of functional annotation and clustering of the gene list, DAVID 6.8 (The Database for Annotation, Visualization and Integrated Discovery v6.8) [9, 16] was used. The functional features used were conversion of gene ids from entrez id to uniprot accession, identifying enriched biological processes, molecular functions, cellular compartmental localization of the genes. In order to understand statistical significance of the enriched terms or enriched pathways, the integrated analysis in terms of p-value and FDR was considered as the statistical parameter to build confidence. For the purpose of uniformity, across different gene sets, all were converted to human uniprot accessions.

2.4.Protein-Protein Interaction networks

To check for any kind of interaction and connection amongst each gene, the set of genes were submitted to STRING v10.5 as batch query and the resultant network was analyzed for several

network parameters such as number of nodes, number of edges, average node degree, average local clustering coefficient and the enrichment of the network. The connection between two interaction partners will be established if the two genes are already known to interact from databases or based on predictive interactions such as gene neighborhood, gene-gene fusions and gene co-occurrences.

3. RESULTS

3.1. Finding the set of differentially expressed proteins for different cancers

For each type of cancers, several databases were explored that carries repository of data from different independent screens related to differentially expressed genes and proteins. The preliminary search for databases was done using PubMed to find reviews such as Pavlopoulou et al. (2015) which can provide us a comprehensive list of datasets and databases publicly available for such kind of analysis [8]. After thorough scrutiny, for this work, the data made available in dbDEPC3.0 database was used. However, before doing the analysis, pooling of certain cancer subgroups and subtypes were done to prepare a simple cumulative list. This has also led to the exclusion of certain cancer subtypes from the analysis which presented a different pathophysiology. Such cancers were pooling was done were Brain, Breast, Glioma, Head and Neck, Hepatocellular carcinoma, Leukemia, Lung, Lymphoma, Pancreatic, Sarcoma, Skin and Thyroid. Moreover, the excluded cancer subtypes were Triple Negative Breast cancer, Oral premalignant Head and Neck cancer and Small Cell Lung Carcinoma.

Furthermore, a total of 25 cancer groups were screened within dbDEPC3.0, each carrying set of differently expressed genes from 4 different study designs, namely, Normal vs Cancer (N_vs_C), Cancer vs Cancer (C_vs_C), Treatments (none, sensitive, resistant) and Metastatic group. The same can be visualized in Fig1A wherein cumulative distribution of all the DEPs (Differentially Expressed Proteins) is shown for different cancer types and Fig1B wherein the number of each DEPs for each cancer group were categorized as per the 4 different study groups. It was evident that data for not all the cancer groups was available for each cancer groups. Also, the distribution reflected variations because of the underlying experimental design from which the gene was obtained.

3.2. Analysis of major cancer groups with distribution of queries across all 4 categories.

After manually curating through each cancer group to look at the distribution of queries across all 4 categories, we selected 11 major cancer groups namely Adenocarcinoma, Breast cancer, Cervical carcinoma, Colorectal cancer, Gastric cancer, Hepatocellular carcinoma, Head and Neck cancer, Lung cancer, Ovarian cancer, Prostate cancer and Urinary bladder cancer. In each of the cancer type, we attempted to find common set of genes shared amongst categories. As seen in Fig2; amongst the different overlaps, the number of shared genes was not significant in Adenocarcinoma, Prostate cancer and Urinary bladder cancer. However, in the case of Cervical, Gastric, Lung and Ovarian cancer significant sharing of genes was observed in only certain categories (i.e. between certain categories). Notably, in Breast, Colorectal and HNC presence of shared genes was found amongst all 4 categories. These analyses indicate that for each cancer type, the study design has a major influence in the distribution of genes shared amongst different categories. Moreover, in some

cancers, the absence of sharing is due to the lack of sufficient quantity of experimental data. However, for the purpose of further analysis we will restrict our study to most prominent female cancers such as Breast, Cervical and Ovarian with a comparative reference of Colorectal cancer.

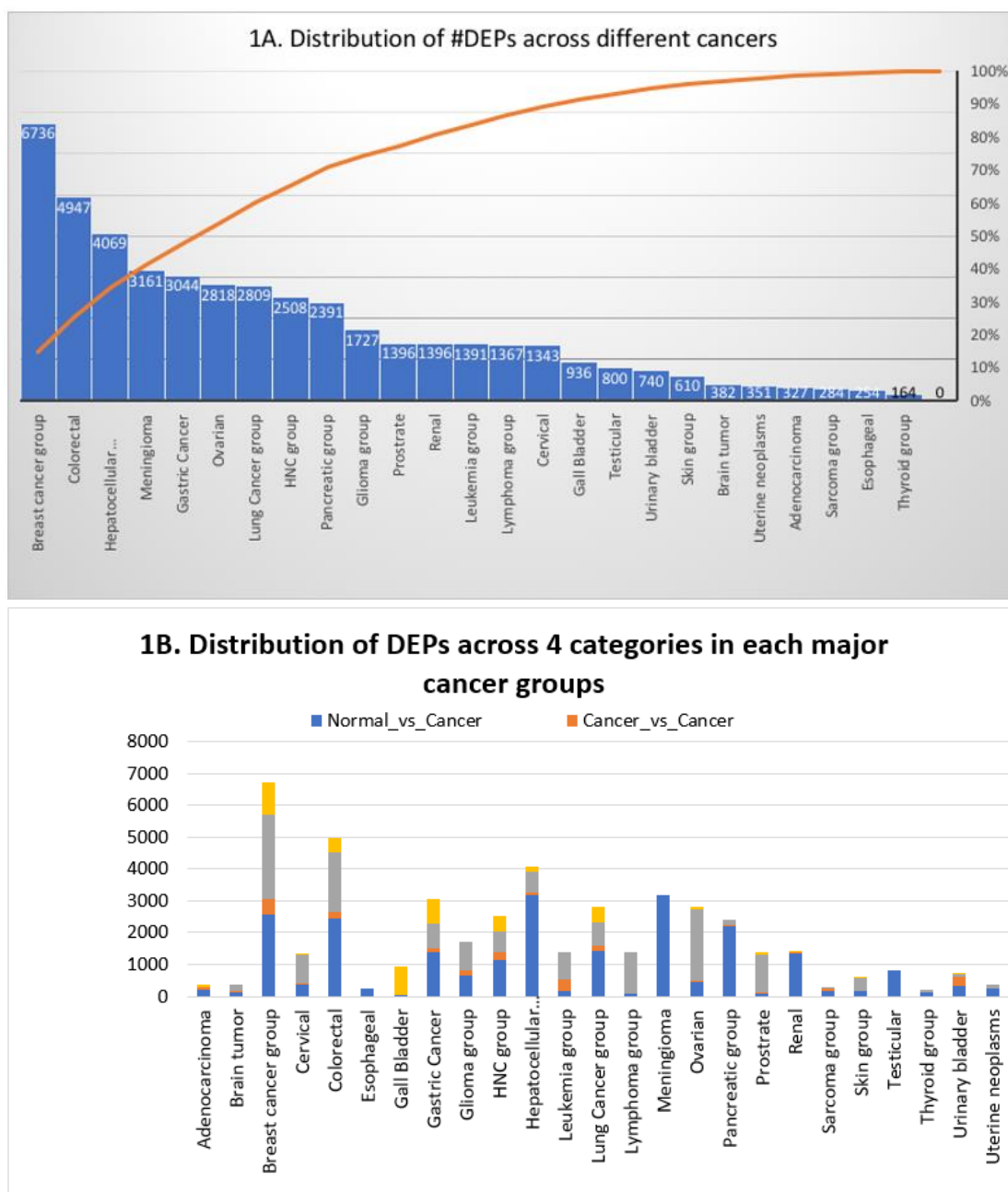


Figure 1: Downloading of data from the dbDEPC3.0 (A) The distribution of cumulative number of DEPs (differentially expressed proteins is represented from maximum to minimum (left to right) in a particular cancer on X-axis and (B) The distribution of DEPs in each cancer group belonging to the 4 study designs: Normal vs Cancer, Cancer vs Cancer, Treatment groups and Metastatic groups. Note that not all the cancer has distribution of DEPs in all 4 categories.

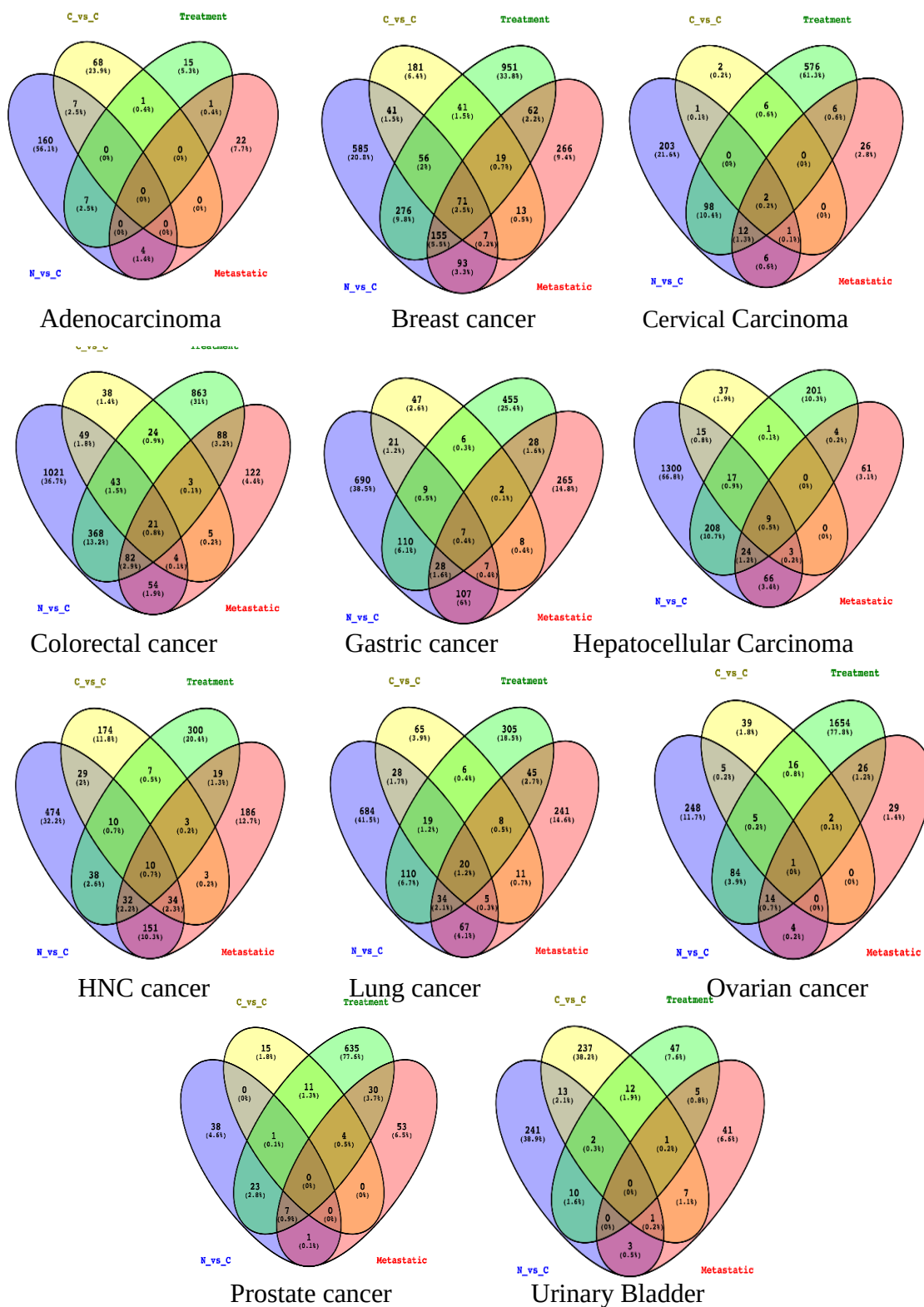
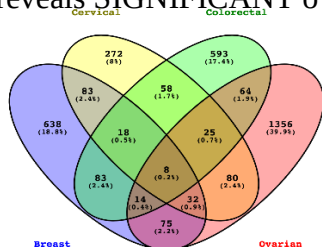


Figure 2: Analysis of 11 major cancer types for finding shared genes among different categories using Venny3.0

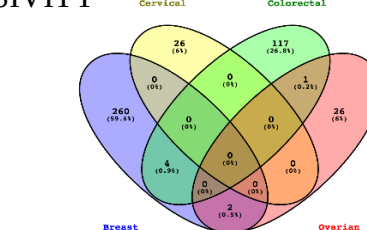
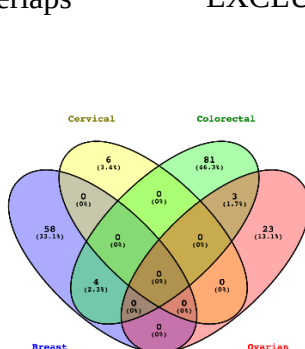
3.3. Analysis of common and exclusive signatures of Treatment and Metastatic groups

Since the aim of this study is to identify non-oncogenic support systems in cancer, we aimed our analysis at identifying any common signatures among the treatment groups of breast, cervical, colorectal and ovarian cancers. For this purpose, the treatment exclusive groups of each individual cancer types were taken and compared against each other (Fig.3A). Similarly, the metastatic exclusive groups of each individual cancer types were taken and compared against each other (Fig.3B). Another small comparison was also carried out between the common genes of treatment and metastatic groups from each individual cancer types to see if there is any sharing of such genes as well (Fig.3C). It was observed that there is substantial overlap amongst the ‘Treatment exclusive’ category between 4 different cancers. On the contrary, the genes were not found to overlap in the ‘Metastatic exclusive’ and ‘Treatment and Metastatic commons’ group. For the ‘Treatment exclusive’ group; 83 common elements were found in Breast and Cervical cancer, 83 common elements were found in Breast and Colorectal cancer, 75 common elements were found in Breast and Ovarian cancer, 58 common elements in Cervical and Colorectal cancer, 80 common elements in Cervical and Ovarian cancer, 64 common elements in Colorectal and Ovarian cancer were found. For the triad groups, 18 common elements in Breast, Cervical and Colorectal cancer, 14 common elements in Breast, Colorectal and Ovarian cancer, 25 common elements in Cervical, Colorectal and Ovarian cancer, 32 common elements in Breast, Cervical and Ovarian cancer were found. 8 common elements were found in Breast, Cervical, Colorectal and Ovarian cancers. For further analysis, we chose to proceed with the 8 common elements (P00491, Q6YN16, P28070, Q15436, P46783, P62888, P67809, P22392) in Breast, Cervical, Colorectal and Ovarian cancers. We also chose to look into the 32 common elements (P262263, P30050, P61531, Q14847, Q15942, Q9Y230, P51571, Q7KZF4, P26373, P36871, P37108, P49207, P49591, P505502, P61353, P62424, P62829, P62906, Q15056, Q8IY81, Q9BX68, P46781, P83731, P62277, P61313, P38159, Q15233, P39656, Q6P2Q9, Q9NX63, O95373, P26640) in Breast, Cervical and Ovarian cancer as they represent a female cancer specific group which are all hormone responsive.

3A. Analysis of Treatment only groups between different cancers reveals SIGNIFICANT overlaps



3B. Analysis of Metastatic only groups between different cancers reveal EXCLUSIVITY



3C. Analysis of commons of Treatment and Metastatic groups between different cancers reveals NO SIGNIFICANT common interactions

Figure 3: Analysis of Treatment and Metastatic elements in Breast, Cervical, Colorectal and Ovarian Cancers.

3.4.Functional Annotation and Enrichment Analysis of Treatment group

The 8 common elements found to be shared between Breast, Cervical, Colorectal and Ovarian cancer and 32 common elements in Breast, Cervical and Ovarian cancer from the ‘Treatment exclusive’ groups were analyzed for functional annotation and enrichment analysis preliminarily through dbDEPC3.0 and through DAVID 6.8. For the 8 common elements, 2 annotation clusters were formed and for the 32 elements, 7 annotation clusters were formed. Enrichment analysis reveals 3 important pathways GO: 0070062 - extracellular exosome related processes, GO: 0006614 - SRP-dependent cotranslational protein targeting to membrane and GO: 0005925 - focal adhesion where most of the annotated genes were mapped (Fig.4A, 4C). These genes were also analyzed for changes in fold expression in treatment and/or resistant groups (Fig.4B, 4D) and it was seen that the pattern of change in fold expression in most of these candidates is opposite in treatment v/s resistant groups of a particular type. For eg. : Candidate P46783 and P62888 expressed no change in fold expression in the treatment groups of breast and cervical cancer whereas they were found to be down-regulated in resistant groups of breast and cervical cancer (Fig.4B). Similarly, candidate P28070 showed significant upregulation in the fold-change expression in resistant groups of breast and cervical cancers. Similarly, 3 candidates P30050, P62263, P26373 were significantly up-regulated in breast cancer treatment group and down-regulated in breast cancer resistant group (Fig. 4D). Candidates P49207, P61353, P30050, P62424, P62906, P61313 and P46781 were found to be down-regulated in both breast cancer and cervical cancers showing similar patterns of change in fold expressions (Fig. 4D). For the candidates enriched in the category GO: 0006614 - SRP-dependent cotranslational protein targeting to membrane and GO: 0005925 - focal adhesion, protein-protein interactions were analyzed by submitting the query to STRING database. An enriched pathway ($p < 1.0 \times 10^{-16}$) emerged from the submitted query in which the no. of edges was found to be significantly higher than expected (150 vs 19) around 32 nodes. Analysis of different common and exclusive DEPs in different overlapping Treatment groups of Breast, Colorectal, Cervical and Ovarian Cancers is in Figure 4 (A-E).

Annotation Cluster 1 Enrichment Score: 1.5770906066326393								
Category	Term	Count	%	P-Value	Genes	List Total	Fold Enrichment	FDR
GOTERM_CC_DI RECT	GO:0070062~extracellular exosome	6	75	0.001390	P46783, P00491, P22392, P28070, P62888, P67809	8	4.862	1.2568
GOTERM_CC_DI RECT	GO:0005737~cytoplasm	5	62.5	0.109223	P00491, P22392, P28070, P62888, P67809	8	2.181	65.0697
GOTERM_CC_DI RECT	GO:0005634~nucleus	5	62.5	0.122310	P00491, P22392, P28070, P62888, P67809	8	2.103	69.4686

Figure 4A: Analysis of common signatures across all 4 cancers. Functional Annotation Clustering using DAVID6.8 identifies 2 major clusters of which 1 is presented here and 75 % of all genes to be strongly associated with GO term GO: 0070062 - extracellular exosome related processes.

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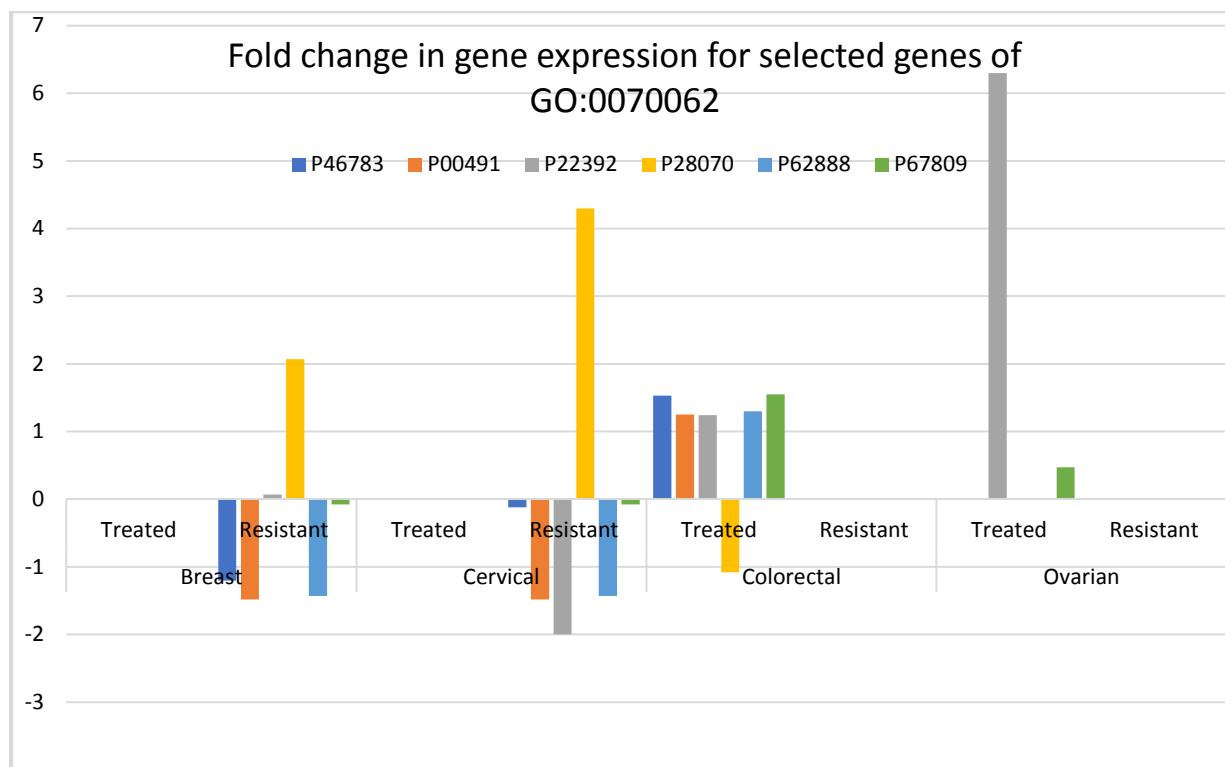


Figure 4B: Comparative analysis of fold-change in expression levels between treatment and resistant groups. The selected genes of GO: 0070062 were retrospectively analyzed from the database and their change in expression within treatment and resistant group was compared for breast, cervical, colorectal and ovarian cancers.

Annotation Cluster 1		Enrichment Score:						
		15.7423425700168						
Category	Term	Count	%	P-Value	Genes	List Total	Fold Enrichment	FDR
GOTERM_BP_DIRECT	GO:0006614 - SRP-dependent cotranslational protein targeting to membrane	14	43.75	2.46E-22	P61513, P49207, P83731, P62277, P61353, P30050, P62424, P62906, P37108, P62829, P61313, P62263, P26373, P46781	31	80.675	2.86E-19
UP_KEYWORDS	Ribonucleoprotein	16	50	3.94E-20	P61513, P49207, P83731, P62277, P61353, P30050, P62424, P37108, P62906, P62829, Q6P2Q9, P61313, P62263, P26373, P46781, P38159	32	34.765	4.28E-17
GOTERM_BP_DIRECT	GO:0006413 - translational initiation	14	43.75	4.18E-20	P61513, P49207, P83731, P62277, P61353, P30050, Q15056, P62424, P62906, P62829, P61313, P62263, P26373, P46781	31	55.353	4.86E-17
GOTERM_BP_DIRECT	GO:0019083 - viral transcription	13	40.625	3.28E-19	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	31	62.873	3.82E-16

GOTERM_BP_DIRECT	GO:0000184 - nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	13	40.625	7.00E-19	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	31	59.174	8.14E-16
UP_KEYWORDS	Ribosomal protein	13	40.625	2.33E-17	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	32	45.194	2.54E-14
GOTERM_BP_DIRECT	GO:0006412 - translation	14	43.75	1.42E-16	P61513, P49207, P83731, P62277, P61353, P30050, P62424, P62906, P62829, P49591, P61313, P62263, P26373, P46781	31	29.974	1.33E-13
GOTERM_BP_DIRECT	GO:0006364 - rRNA processing	13	40.625	9.36E-16	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	31	32.905	1.03E-12
GOTERM_MF_DIRECT	GO:0044822 - poly(A) RNA binding	20	62.5	1.07E-15	P61513, Q8IY81, P83731, P62277, P61353, Q15942, P30050, Q15233, Q15056, Q7KZF4, P62424, P37108, P62906, P62829, Q6P2Q9, P61313, P62263, P26373, P46781, P38159	31	9.646	1.18E-12
GOTERM_MF_DIRECT	GO:0003735 - structural constituent of ribosome	13	40.625	1.37E-15	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	31	31.887	1.41E-12
GOTERM_CC_DIRECT	GO:0022625 - cytosolic large ribosomal subunit	10	31.25	1.52E-15	P61513, P49207, P62424, P62906, P83731, P62829, P61353, P61313, P26373, P30050	32	83.75	1.60E-12
GOTERM_CC_DIRECT	GO:0005840 - ribosome	12	37.5	1.83E-15	P61513, P49207, P62424, P62906, P62277, P83731, P62829, P61353, P61313, P62263, P26373, P46781	32	41.168	1.82E-12
KEGG_PATHWAY	hsa03010:Ribosome	13	40.625	2.29E-15	P61513, P49207, P62277, P83731, P61353, P30050, P62424, P62906, P62829, P61313, P62263, P26373, P46781	24	27.521	1.82E-12
GOTERM_CC_DIRECT	GO:0005925 - focal adhesion	12	37.5	2.22E-11	P61513, P50552, P62424, P62906, P62277, P62829, P61353, Q14847, Q15942, P62263, P46781, P30050	32	17.478	2.28E-08
GOTERM_CC_DIRECT	GO:0005829 - cytosol	20	62.5	1.22E-07	P61513, P49207, P83731, P62277, P61353, P30050, P26640, Q15056, O95373, P50552, P36871, P62424, P37108, P62906, P62829, P49591, P61313, P62263, P26373, P46781	32	3.435	1.25E-04

Figure 4C: Analysis of common signatures between Breast, Cervical and Ovarian Cancer. Functional Annotation Clustering using DAVID6.8 identifies 7 major clusters of which 1 is presented here and in which 14/31 genes to be significantly associated with GO term GO: 0006614- SRP-dependent cotranslational protein targeting to membrane

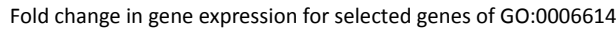


Figure 4D: Comparative analysis of fold-change in expression levels between treatment and resistant groups. The selected genes of GO: 0006614 were retrospectively analyzed from the database and their change in expression within treatment and resistant group was compared for breast, cervical, colorectal and ovarian cancers.

PPI enrichment p-value: $<1.0e-16$ | Number of nodes: 32 | Expected no. of edges: 19
Number of edges found: 150 | Average node degree: 9.38 | Avg. local clustering co-eff: 0.659

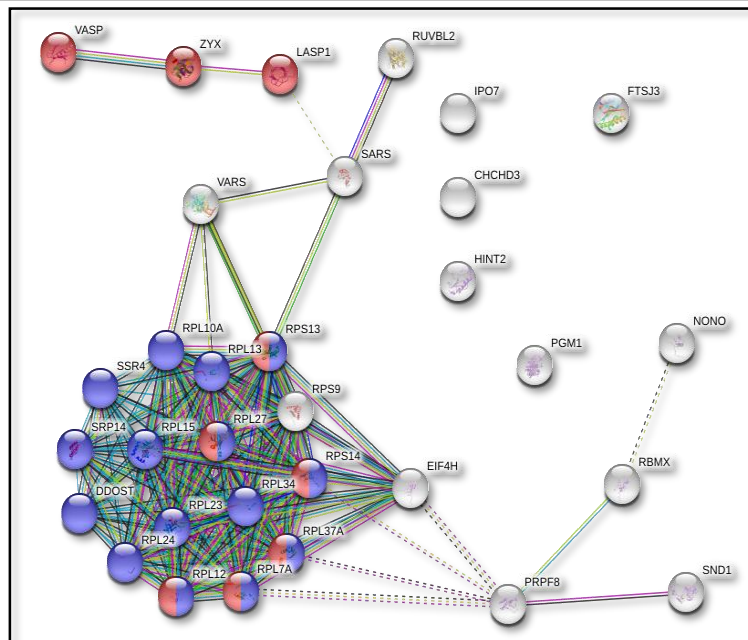


Figure 4E: Analysis of these 32 candidates in STRING database shows functional enrichment in the protein-protein interaction with p-value: $<1.0e-16$. Proteins involved in **GO: 0006614 (#15)** are marked in Blue and Proteins with **GO: 0005925** related to the function of focal adhesion (#9) are marked in Red.

3.5.Functional Annotation and Enrichment Analysis of Metastatic group

The exclusive elements found non-overlapping between Breast, Cervical, Colorectal and Ovarian cancer were mapped for functional annotation and clustering. Analysis of metastatic signatures exclusive to breast cancer group (Fig.5A) for functional annotation using DAVID 6.8 identifies 48 clusters out of which the most significant is shown here. Within the cluster, 30 elements have been mapped to GO: 0005913~cell-cell adherens junction, 29 elements have been mapped to GO: 0098641 - cadherin binding involved in cell-cell adhesion and 15 elements have been mapped to GO: 0098609 - cell-cell adhesion. Analysis of metastatic signatures exclusive to cervical cancer group (Fig.5B) for functional annotation using DAVID 6.8 identifies 5 clusters out of which the most significant is shown here. Within the cluster, 9 elements have been mapped to GO: 0005615 - extracellular space.

Analysis of metastatic signatures exclusive to colorectal cancer group (Fig.5C) for functional annotation using DAVID 6.8 identifies 26 clusters out of which the most significant is shown here. Within the cluster, 24 elements have been again mapped to GO: 0005615 - extracellular space. Analysis of metastatic signatures exclusive to ovarian cancer group (Fig.5D) for functional annotation using DAVID 6.8 identifies 5 clusters out of which the most significant is shown here. Within the cluster, 5 elements have been mapped to GO: 0003779 – actin binding.

By carrying out protein-protein interaction analysis using STRING, no significant network enrichment was obtained in any of the category and most of the candidates were found to be disconnected to other proteins. This analysis revealed that the progression of metastasis in these cancer types may recruit specific candidates or may go via different pathways. Analysis of different exclusive DEPs in Metastatic groups of Breast, Colorectal, Cervical and Ovarian Cancers is in Figure 5 (A-D).

Annotation Cluster 1		Enrichment Score:							
		16.882966332807833							
Category	Term	Count	%	P-Value	Genes	List Total	Fold Enrichment	FDR	
GOTERM_CC_DIR ECT	GO:0005913 - cell-cell adherens junction	30	12.30	3.36E- 22	P07356, Q99P72, P63017, Q61166, P05784, Q62523, O08709, Q9WVK4, Q8VDD5, Q9QXS1, Q62261, P24452, P62855, P62827, P09055, P20029, Q9JLV1, Q9CY58, P26039, P11499, P06151, Q9WVA4, P05064, P62962, Q9CWJ9, Q61553, O35685, Q80X90, Q78PY7, P10107	163	11.452	4.56E-19	
GOTERM_MF_DI RECT	GO:0098641 - cadherin binding involved in cell-cell adhesion	29	11.89	4.40E- 21	P07356, Q99P72, P63017, Q61166, P05784, O08709, Q9WVK4, Q8VDD5, Q9QXS1, Q62261, P24452, P62855, P62827, P09055, P20029, Q9JLV1, Q9CY58, P26039, P11499, P06151, Q9WVA4, P05064, P62962, Q9CWJ9, Q61553, O35685, Q80X90, Q78PY7, P10107	164	11.057	6.18E-18	
GOTERM_BP_DIR ECT	GO:0098609 - cell-cell adhesion	15	6.15	1.52E- 09	Q9JLV1, Q9CY58, P26039, P06151, Q9WVA4, P05064, O08709, Q9CWJ9, Q9QXS1,	162	8.859	2.46E-06	

					Q61553, Q80X90, O35685, Q78PY7, P24452, P62855			

Figure 5A: Analysis of metastatic signatures exclusive to Breast cancer group. Functional Annotation Clustering using DAVID 6.8 identifies 48 major clusters of which 1 is presented below. The most significant association was found to be with 3 different GO categories GO: 0005913 - cell-cell adherens junction, GO: 0098641 - cadherin binding involved in cell-cell adhesion and GO: 0098609 - cell-cell adhesion.

Annotation Cluster 1 Enrichment Score: 1.5711064669654715								
Category	Term	Count	%	P-Value	Genes	List Total	Fold Enrichment	FDR
GOTERM_CC_DIRECT	GO:0005615 - extracellular space	9	50.000	1.16E-05	G5E9R0, P68104, O94964, P12110, P01857, P20231, P05109, Q05707, Q15661, P08709	18	6.765	0.0117
UP_KEYWORDS	Secreted	8	44.444	5.87E-04	O94964, P12110, P01857, P20231, P05109, P31151, Q05707, Q15661, P08709	18	4.655	0.6258
GOTERM_CC_DIRECT	GO:0005576 - extracellular region	7	38.889	0.00248	P12110, P01857, P20231, P05109, P31151, Q05707, Q15661, P08709	18	4.402	2.4832
UP_KEYWORDS	Hydroxylation	3	16.667	0.00274	P12110, Q05707, P08709	18	36.107	2.8894
GOTERM_CC_DIRECT	GO:0005788 - endoplasmic reticulum lumen	3	16.667	0.01353	P12110, Q05707, P08709	18	15.819	12.8882
GOTERM_MF_DIRECT	GO:0004252 - serine-type endopeptidase activity	3	16.667	0.02373	P01857, P20231, Q15661, P08709	17	11.682	21.2504
GOTERM_CC_DIRECT	GO:0031012 - extracellular matrix	3	16.667	0.03045	P12110, P20231, Q05707, Q15661	18	10.261	26.8840
UP_SEQ_FEATURE	signal peptide	5	27.778	0.31169	P12110, P01857, P20231, Q05707, Q15661, P08709	18	1.666	98.5991
UP_KEYWORDS	Disulfide bond	5	27.778	0.31202	P01857, P20231, P31151, Q05707, Q15661, P08709	18	1.665	98.1599
UP_SEQ_FEATURE	disulfide bond	4	22.222	0.45840	P01857, P20231, P31151, Q15661, P08709	18	1.528	99.9095
UP_SEQ_FEATURE	glycosylation site: N-linked (GlcNAc...)	5	27.778	0.49696	P12110, P01857, P20231, Q05707, Q15661, P08709	18	1.316	99.9611
UP_KEYWORDS	Glycoprotein	5	27.778	0.53788	P12110, P01857, P20231, Q05707, Q15661, P08709	18	1.256	99.9738
UP_KEYWORDS	Signal	4	22.222	0.69806	P12110, P20231, Q05707, Q15661, P08709	18	1.099	99.9997

Fig-5B: Analysis of metastatic signatures exclusive to cervical cancer group. Functional Annotation Clustering using DAVID 6.8 identifies 5 major clusters of which 1 is presented below. The most significant association was found to be with GO category GO: 0005615 - extracellular space with 9 hits.

Annotation Cluster 1	Enrichment Score: 2.3497959351							
Category	Term	Count	%	P-Value	Genes	List Total	Fold Enrichment	FDR
GOTERM_CC_DIRECT	GO:0005615 - extracellular space	24	20.51	8.41E-06	P10646, P13987, P02753, Q96DA0, Q16674, P41273, Q92876, O14944, O76061, P04040, P43652, P00751, P55145, P35241, Q9BWS9, Q9BY44, P24592, P02656, Q6X4U4, P41271, Q14563, O15240, P01037, P55058	115	2.8235	0.011
UP_SEQ_FEATURE	signal peptide	37	31.62	9.53E-05	P10646, P13987, P02753, Q96DA0, Q16674, Q9BVK6, Q16769, O14944, O76061, Q9H9K5, Q99435, Q5FWE3, P00751, Q9BWS9, Q7Z7H5, P02656, P08174, P41271, O15240, P55058, P51571, Q6UXI9, Q92876, P43652, P55145, P54764, P04156, Q92859, Q9BVC6, P24592, Q9HCN8, Q6X4U4, P52797, O95445, Q9Y3B3, P01037, Q14563	117	1.8962	0.135
UP_KEYWORDS	Secreted	25	21.37	2.17E-04	P10646, P13987, P02753, Q96DA0, Q16674, Q92876, O14944, Q16769, O76061, P43652, Q99435, P55145, P00751, Q9BWS9, P24592, P02656, Q6X4U4, P08174, P41271, O95445, P01037, O15240, Q14563, P55058, Q6UXI9	117	2.2380	0.280
UP_KEYWORDS	Signal	39	33.33	0.00100	P10646, P13987, P02753, Q96DA0, Q16674, Q9BVK6, Q16769, O14944, O76061, Q9H9K5, Q99435, Q5FWE3, P00751, Q9BWS9, Q7Z7H5, P02656, P08174, P41271, O15240, P55058, P51571, Q6UXI9, Q99720, Q9UPQ7, Q92876, P43652, P55145, P54764, P04156, Q92859, Q9BVC6, P24592, Q9HCN8, Q6X4U4, P52797, O95445, Q9Y3B3, P01037, Q14563	117	1.6491	1.283
UP_KEYWORDS	Disulfide bond	29	24.79	0.02480	P10646, P13987, P02753, Q16674, Q16769, O14944, O76061, Q99435, P00751, P41271, P08174, P55058, P51571, Q6UXI9, P47985, Q92876, Q9BYC5, P43652, Q9Y662, P55145, P04156, Q92859, Q9UKM7, P24592, Q6X4U4, P52797, O95445, Q14563, P01037	117	1.4855	27.619
UP_SEQ_FEATURE	disulfide bond	26	22.22	0.02620	P10646, P13987, P02753, Q16674, O14944, Q99435, P00751, P08174, P41271, P55058, P51571, Q6UXI9, P47985, Q92876, P43652, Q9Y662, P55145, P04156, Q9UKM7, Q92859, P24592, Q6X4U4, P52797, O95445, P01037, Q14563	117	1.5284	31.356
GOTERM_CC_DIRECT	GO:0005576 - extracellular region	16	13.68	0.07690	P10646, P02753, Q92876, O14944, O76061, P43652, Q99435, P00751, P24592, P02656, P52797, P08174, O95445, Q14563, P55058, Q6UXI9	115	1.5749	63.836
UP_KEYWORDS	Glycoprotein	30	25.64	0.25742	P10646, P13987, Q96DA0, Q9BVK6, Q16769, O14944, O76061, Q9H9K5, Q99435, Q5FWE3, P00751, P02656, Q7Z7H5, P08174, Q7Z5M5, P55058, Q9NPF2, Q92876, P43652, Q9Y662,	117	1.1596	97.832

					P55145, P54764, P04156, Q92859, P24592, Q6X4U4, P52797, Q95445, Q9Y3B3, Q14563			
UP_SEQ_FEATURE	glycosylation site: N-linked (GlcNAc...)	28	23.93	0.31639	P10646, P13987, Q96DA0, Q9NPF2, Q9BVK6, Q92876, O14944, Q16769, O76061, P43652, Q99435, Q9H9K5, Q5FWE3, Q5T3F8, Q9Y662, P54764, P00751, P04156, Q92859, Q7Z7H5, Q6X4U4, P52797, P08174, Q95445, Q9Y3B3, Q14563, Q7Z5M5, P55058	117	1.1340	99.544

Fig-5C: Analysis of metastatic signatures specific to Colorectal cancer group. Functional Annotation Clustering using DAVID 6.8 identifies 26 major clusters of which the most significant one is presented below. The strongest association was found to be with GO category GO:0005615 - extracellular space with 9 hits.

Annotation Cluster 1	Enrichment 2.2440059516605606	Score:						
Category	Term	Count	%	PValue	Genes	List Total	Fold Enrichment	FDR
UP_KEYWORDS	Actin-binding	5	19.23	3.12E-04	Q99439, P09493, Q05682, Q60FE7, Q8N8S7	26	14.445	0.3541
GOTERM_MF_DIRECT	GO:0003779~actin binding	5	19.23	6.93E-04	Q99439, P09493, Q05682, Q60FE7, Q8N8S7	26	11.678	0.7482
GOTERM_CC_DIRECT	GO:0005856~cytoskeleton	5	19.23	0.001524	Q99439, P09493, Q05682, Q14764, Q8N8S7	26	9.446	1.6417
GOTERM_CC_DIRECT	GO:0005925~focal adhesion	5	19.23	0.001847	Q99439, P24821, P05362, Q60FE7, Q8N8S7	26	8.963	1.9868
GOTERM_CC_DIRECT	GO:0001725~stress fiber	3	11.54	0.002475	Q99439, P09493, Q60FE7	26	38.940	2.6540
GOTERM_BP_DIRECT	GO:0098609~cell-cell adhesion	4	15.38	0.007353	Q99439, Q05682, Q60FE7, P42167	26	9.533	9.6538
GOTERM_MF_DIRECT	GO:0098641~cadherin binding involved in cell-cell adhesion	4	15.38	0.008725	Q99439, Q05682, Q60FE7, P42167	26	8.955	9.0550
GOTERM_CC_DIRECT	GO:0005913~cell-cell adherens junction	4	15.38	0.009504	Q99439, Q05682, Q60FE7, P42167	26	8.680	9.8470
UP_KEYWORDS	Cytoskeleton	5	19.23	0.046541	P09493, Q05682, Q60FE7, Q14194, Q8N8S7	26	3.478	41.8418
GOTERM_CC_DIRECT	GO:0005886~plasma membrane	5	19.23	0.849797	Q05682, P05121, Q60FE7, Q8N8S7	26	0.850	100.0000

Fig-5D: Analysis of metastatic signatures specific to Ovarian cancer group. Functional Annotation Clustering using DAVID 6.8 identifies 5 major clusters of which the most significant one is presented below. The strongest association was found to be with GO category GO: 0003779 – actin binding with 5 hits.

4. DISCUSSION

The work we present here, unveils previously found yet undiscovered candidates in different independent screens. Our analysis across 779 MS experiments by different groups helped

understand common signatures found in cancers of breast, cervix, colorectal and ovaries. It is known that targeted therapies lead to acquired resistance and hence continuously warrants discovery of better and efficient targets. Our work highlights some important pathways that probably contributes in determining the outcome of treatment modality or development of resistance. This is highlighted by our finding of common elements P00491, Q6YN16, P28070, Q15436, P46783, P62888, P67809, P22392 regulating treatment modalities in breast, cervical, colorectal and ovarian cancers. As has been shown in this work, 6 out of 8 elements from this group have been related to extracellular exosome related process which may have a role to play in the way cell behaves to therapeutic drugs (uptake or recycling).

Similarly, the elements P262263, P30050, P61531, Q14847, Q15942, Q9Y230, P51571, Q7KZF4, P26373, P36871, P37108, P49207, P49591, P505502, P61353, P62424, P62829, P62906, Q15056, Q8IY81, Q9BX68, P46781, P83731, P62277, P61313, P38159, Q15233, P39656, Q6P2Q9, Q9NX63, O95373, P26640 were found to be common amongst breast, cervical and ovarian cancer in regulating treatment modalities. Functional enrichment of these candidates highlighted GO: 0006614 - SRP-dependent cotranslational protein targeting to membrane and GO: 0005925 - focal adhesion pathways. Protein-protein interactions indicate the need of mapping many newly discovered candidates to the network. We also believe that the 3 candidates (VASP, ZYX, LASP-1) discovered through this meta-analysis may play important role and uncover mechanisms in which cancer cells responds to therapies. While trying to find similarities in Metastatic signatures, we have found no significant overlap in candidates across the 4 cancer types. This could have been in part due to distinct mechanisms employed by the cells in undergoing metastasis in these cancers, recruitment of distinct candidates during the process or in part due to availability of only limited data from the screens we studied [17, 18, 19, 20]. We therefore do not neglect the possibility of finding newer interactions and candidates should more datasets be included in the study and confirmatory experiments be carried out with specific genetic cancer models [24, 25, 26, 27]. However, our work paves way to corroborating data form different screens and different platforms and therefore giving more insight into previously found but undiscovered candidates.

5. CONCLUSION

We present a simple approach for corroborating and collaborating enormous data available on public resources with the aim of discovering new candidates having role in therapeutic or resistance development. These new candidates and pathways could emerge to support the oncogenic signaling pathways and hence may serve as potential targets. However, there is still a pressing need to validate our data with animal model and screening of clinical specimens in a controlled patient population.

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Identification of Optimal Conditions for Developing an Effective and Commercially Viable Biologic Product for Human Use out of Dental Pulp Stem Cell Conditioned Medium

R. MOKASHI*, D. DASGUPTA

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, India.

ABSTRACT: Revolutionary strides have been made in stem cell research however translation of research into therapeutic application has been relatively sluggish for multitude of reasons ranging from high costs to stringent regulations. Hence, stem cell derived products like conditioned medium are gaining fast inroads especially in areas where stem cells are known to act through paracrine effect as they are able to address the reigning challenges associated with stem cells. Stem cell-derived conditioned medium hold a promising prospect to be produced as pharmaceuticals for regenerative medicine. Preliminary studies suggest that dental pulp stem cell (DPSCs) derived conditioned medium is a rich source of growth factors/cytokines, similar to mesenchymal stem cell secretome. Further on, the use of DPSCs as a source for producing conditioned medium scores over other sources from a commercial and therapeutic front because of ease in availability and the proven secretion of the necessary growth factors & cytokines. However, low concentrations of growth factors/cytokines and the high cost for developing the medium especially from mesenchymal stem cells have been major deterrents to development. The current research undertakes an exploratory study to identify the optimal culture conditions beyond hypoxia, considering commercial viability, to get optimal yields of selective cytokines. The derived stem cell conditioned medium using the identified optimal conditions holds strong promise to be developed as a biological active for various healthcare ailments.

KEYWORDS: Dental stem cells, conditioned medium, growth factors, cytokines

***Corresponding Author: Ms. R. Mokashi**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: rajashri.mokashi@gmail.com

1. INTRODUCTION

Regenerative Medicine represents a new paradigm in human health with potential to resolve the unmet need by addressing the root cause of the problem. As per the report from Allied Market Research, Regenerative Medicine is expected to reach \$67.5 billion by 2020 with advancements in stem cell technology and increasing incidences of chronic diseases and trauma injuries as key growth influencers. No wonder, stem cell research is growing twice as fast as the world average growth rate in research and nations investing widely in this area. In addition to stem cell transplantation therapies, stem cell derived products like conditioned medium are gaining fast inroads as they address the reigning challenges associated with stem cells especially in therapeutic

areas wherein the stem cell action is attributed to the paracrine effect stimulating the resident stem cells [1]. This has been reinforced by the recent studies, wherein it has been revealed that stem cells bring about the tissue repair due to their ability to secrete trophic factors. Post this, various studies on mesenchymal stem cell-derived secreted factors showed that the secreted factors alone, without the stem cell, may cause tissue repair in various conditions that involved tissue/organ damage. The secreted factors, referred to as secretome, microvesicles, or exosomes can be found in the medium where the stem cells are cultured; thus, the medium got the name conditioned medium (CM) [2]. This has provided a novel therapeutic approach for treatment simplifying the problems associated with administering the stem cells [3 - 9]. Recent studies have demonstrated the therapeutic effects of the stem cell derived conditioned medium. Stem cell-derived conditioned medium has now been considered a promising prospect to be produced as pharmaceuticals for Regenerative Medicine [10, 11]. Mesenchymal stem cells have the distinct advantage of being immunomodulatory as they regulate the immune response and contribute to reparative processes in different pathological conditions [12].

Considering the therapeutic efficacy of the conditioned medium, the level of paracrine factors secreted by different stem cell resources plays an important role on their influences on cell recruitment and tissue repair. Thereby, devising methods to increase the paracrine factors in stem cell CM enough for them to be used for the treatment becomes imperative. While various stem cell sources have been tried to derive the conditioned medium [13], stem cell intrinsic and preliminary studies suggest that dental pulp stem cell (DPSCs) conditioned medium is a rich source of growth factors/cytokines, similar to mesenchymal stem cell secretome and also better than other stem cell sources especially in angiogenesis [14 - 21]. Additionally, the use of DPSCs as a source for producing conditioned medium scores over other sources from a commercial and therapeutic front because of ease in availability and the proven secretion of the necessary growth factors & cytokines. However, there has been no systematic study to identify optimal conditions for developing an effective and commercially viable biologic product for human use out of dental pulp stem cell conditioned medium to get reproducible results for development of a raw material for pharmaceuticals. This research focuses on the identification of the optimal conditions by profiling of selective cytokines under various experimental situations.

2. MATERIALS & METHODS

2.1.Preparation of the dental pulp stem cell conditioned medium

For preparation of the conditioned medium, pooled sample of dental pulp stem cells was cultured at various passages (2-5) in a standard media comprising of MEM, FBS, standard nutrients like glutamine and ascorbic acid under normoxic conditions in a CO₂ incubator at 37°C. On reaching 80-85% cell confluency, the spent media was collected for cytokine analysis. Selective cytokine analysis for Vascular Endothelial Growth Factor (VEGF), Hepatocyte Growth Factor (HGF) and Keratinocyte Growth Factor (KGF) was done using ELISA under different experimental conditions. All the experiments were conducted in triplicates and the average readings were used for analysis.

Materials

MEM – Thermo Fisher Scientific (commercially available)

DMEM- Thermo Fisher Scientific (commercially available)

Primocin – Invivogen (commercially available)

FBS - Thermo Fisher Scientific (commercially available)

PBS - Thermo Fisher Scientific (commercially available)

Trypsin EDTA – 0.5% Thermo Fisher Scientific (commercially available)

Trypan Blue – HiMedia (commercially available)

2.2.Assessment of the cytokines at different passage levels

Pooled sample of Dental pulp stem cells at second passage level (P2) was revived in T25 Flask and cultured by inducing in CO₂ incubator at 37°C. These cells were further passage into passage 4 (P4) and passage 5 (P5) under the standard culture conditions. At 80-85% cell confluency, the spent media was collected from the flask at each of the passage levels (P3, P4 and P5). ELISA analysis was undertaken immediately on the spent media for the selected cytokines.

2.3.Assessment of the cytokines at various culture duration

Pooled sample of Dental pulp stem cells at fifth passage level (P5) was incubated in CO₂ incubator at 37°C, to study the impact of factors at the end of 24hours, 48 hours and 7 days. After the said time points, spent media was collected from the flask and was used for ELISA analysis.

2.4.Impact of serum free media usage on the secretion of cytokines

Pooled sample of Dental pulp stem cells at fifth passage level (P5) was incubated in CO₂ incubator at 37°C. At 80-85% confluency, cells were selected to study the impact of serum free media. Media was discarded and replaced by fresh serum free media (except 20% FBS). The cells were then incubated in the appropriate flask in the CO₂ incubator at 37°C for 48 hours. After 48 hours, the spent media was collected for ELISA analysis.

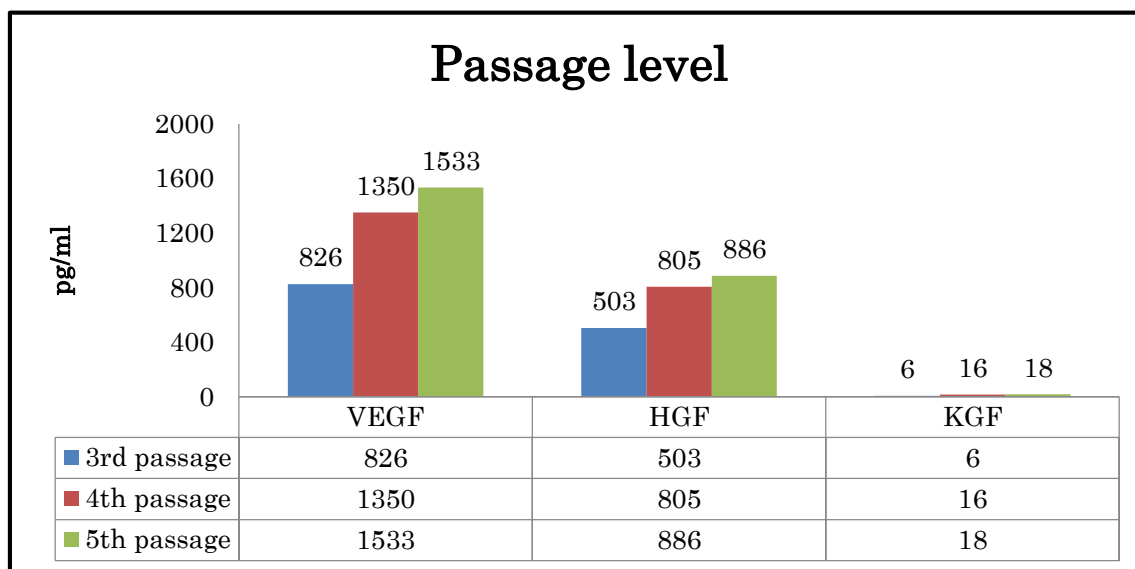
3. RESULTS

3.1.Assessment of the cytokines at various passage levels

There was an up regulation observed in the secretion of studied cytokines with the rise in the passage levels. In absolute value terms, the increase was most pronounced for VEGF, however in percent increase KGF saw the maximum rise. It was also observed that the incremental rise in the cytokine secretion decreased over the passage numbers as evident from the values increase from passage 3 (P3) to passage 4 (P4) and from passage 4 (P4) to passage (P5). Case in point, the percent increase of VEGF from P3 to P4 was 63%, but dropped to 22% from P4 to P5. Similar observations were witnessed in case of HGF and KGF. In fact, the drop was very high in case of KGF although at small base values. This suggests that higher passages necessarily would not result in higher secretions of cytokines and probably one needs to cap to an optimal passage level considering the commercial viability without compromising on yields. VEGF secretions were competitive enough relative to the required therapeutic requirements (~5 ng / ml) considering the current results obtained were sans of use of any ultra-filtration techniques on the spent media. Further on, we

believe that under hypoxic conditions (proven from earlier published studies) will propel the up regulation of the cytokines. Important to note that the cell morphology remained intact through the passages reinforcing the possibility of commercial usage of the dental pulp stem cells at higher passage levels.

(A)



(B)



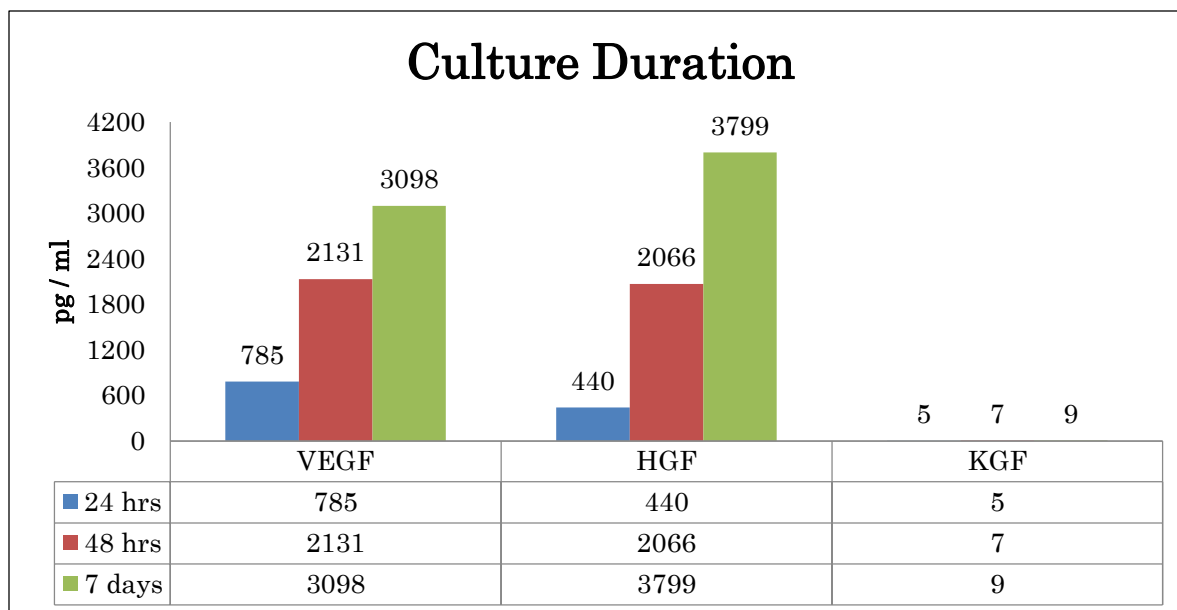
Figure 1: (A) Assessment of selected cytokines; VEGF, HGF and KGF at various passage levels 3rd passage level (P3), 4th passage level (P4) and 5th passage levels (P5) at the end of 48 hours (B) Microscopic images of the dental pulp stem cells at various passage level under the microscope

3.2. Assessment of the cytokines at different culture durations

Culture durations played a very pronounced impact on the secretion of cytokines and there was a linear increase observed across all the cytokines under study. Especially in case of VEGF and HGF, there was a three- fold rise in the cytokine levels from 24 hours to 48 hours and again from 48 hours to 7 days. Mathematically, readings suggest that the rise in the cytokine was more pronounced at 48 hours than from 48 hrs to 7 days suggesting an optimal duration between 48 hours to 72 hours. However in case of KGF, the quantum of cytokine secreted stood at low levels, indicating the need of pre-conditioning factors to boost the secretions in the spent media.

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(A)



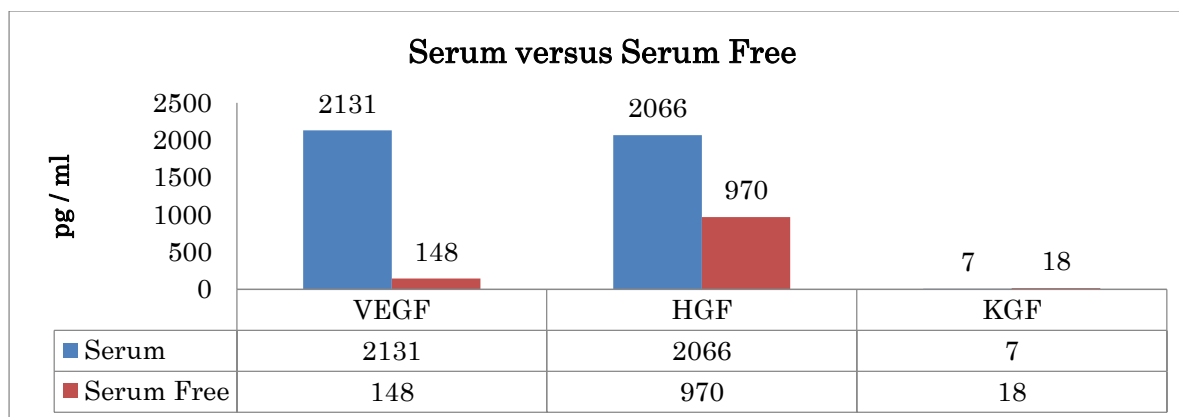
(B)



Figure 2: (A) Assessment of selected cytokines; VEGF, HGF and KGF at different culture duration 24 hours, 48 hours and 7 days at passage 5 (B) Microscopic images of the dental pulp stem cells at different culture duration under the microscope

3.3.Impact of serum free media usage on the secretion of cytokines

Considering the preference for serum free media for therapeutic applications and challenges revolving Fetal Bovine Serum (FBS), it was imperative to evaluate the impact of serum free media usage on the secretion of cytokines. Understandably, cytokine secretions were expected to be lower in serum free media as compared to serum containing media, percent impact was primarily being explored. There was a massive drop in the VEGF and HGF secretions with the use of serum free media while the serum free condition proved beneficial for KGF. The percent drop in the cytokine levels too differed between VEGF and HGF. These findings reinforced the need for pre-conditioning factors and hypoxic conditions to boost the cytokine secretions to compensate for serum.



4. DISCUSSION

Mesenchymal stem cell conditioned media is gaining fast importance owing to its potential of quick translation for number of therapeutic applications. One decisive step in encasing the potential is to have conditioned medium which qualifies the criteria of efficacy, reproducibility and cost effective. It would be mandatory to standardize the production method to ensure that we have a reproducible biological active with known range of cytokine / growth factor content. Further on, it would be critical to identify the factors impacting the secretion of cytokines so that selective ones can be up or down regulated depending on the target indications. While exploring the optimal conditions in this research, efficacy along with commercial viability was considered. Choice of dental pulp stem cells for deriving the conditioned was driven by the fact that dental pulp stem cells score over other sources because of ease in availability and the proven secretion of the necessary growth factors & cytokines. Also dental pulp stem cells have been proven to be stable even at higher passage levels and immuno-modulatory making them cells of choice. To avoid batch to batch variation, this was the first of its kind study conducted on pooled sample of stem cells ensuring better reproducibility in results.

While recent studies have shown hypoxia to induce higher secretions of cytokines, this research has explored other conditions which can play a critical role in increasing the cytokine output. The research has conclusively proven that the cytokine output increases with passage levels and culture duration however the diminishing returns should be taken into consideration while planning out the production process. In a commercial set up, it's most likely that one will have to work at higher passage levels and this research has shown the stability of cells and higher cytokine levels. Also the impact of serum free media on the cytokine secretion directs us towards use of preconditioning factors to compensate for the serum effect.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

The authors hereby declare that they do not have any conflict of interest for the research work communicated through this paper.

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Study of Methylene Tetrahydrofolate Reductase *MTHFR* (C677T) Polymorphism Association with Preterm Delivery

S. PANIKAR¹, S. SHARMA², M. THAKUR^{3*}

¹Department of Medical Genetics, MGMSBS, MGMIHS, Navi Mumbai, India.

²Department of Obstetrics & Gynecology, MGM Medical College, MGMIHS, Navi Mumbai India.

³Department of Medical Biotechnology, MGMSBS, MGMIHS & MGM CET, Navi Mumbai, India.

ABSTRACT: Since decades it has been seen that, gestational age affects the health of an individual. It has also been found that number of infant mortality and morbidity occurs due to preterm deliveries (PTD). The *MTHFR* gene encodes the methylenetetrahydrofolate reductase known to be involved in the homocysteine – methionine pathway. It has been reported that the deficiency of *MTHFR* activity may cause hyperhomocysteinemia which results in adverse pregnancy outcomes. The objective of the present study was to evaluate the associative role of *MTHFR* gene polymorphism in susceptibility to PTD, negative pregnancy outcome and low birth weights (LBW) in Navi Mumbai, Indian population. It was a preliminary study started with 50 PTD cases in which 25 were (extreme preterm (24 - 28 weeks of gestation, n= 3), very preterm (28–<32 weeks of gestation n=7) and late preterm (32–<37 weeks of gestation n=15) and 25 term delivery cases were studied for *MTHFR* (C677T) polymorphism. Distribution of *MTHFR* was higher in PTD cases. Our findings showed presence of only wild type in PCR RFLP and no polymorph were found.

KEYWORDS: *MTHFR*, Preterm delivery, Gestation, Polymorphisms, RFLP

***Corresponding Author: Dr. Mansee Thakur**

Department of Medical Biotechnology, MGMSBS, MGMIHS & MGM CET, Navi Mumbai, India.
E-mail Address: mansibiotech79@gmail.com / mansimgm07@gmail.com

1. INTRODUCTION

Despite all the medical advancement it is still observed, that there is significantly decline in maternal and child health, among them one of the leading medical issues globally found is preterm deliveries. When birth occurs between 20 weeks of pregnancy and 37 weeks of pregnancy, it is called preterm birth [1]. WHO has classified pre term birth (PTB) into three sub categories i.e. extremely preterm (24-28 weeks), very preterm (28 to <32 weeks) and moderately or late pre-term (32 to <37) [2]. Preterm deliveries (PTD) are leading cause of increasing morbidity and mortality among the mother as well as infants including different other health problems [3].

It was analysed globally that 11.1 per 100 live births are preterm. Moreover, there has been variation in incidents' country wise was observed. It varies around 5% in European countries and 15% in sub-Saharan African and Asian population. Every year about 15 million babies are born prematurely around the world and that is more than one in 10 of all babies born globally. Almost

1 million children die each year due to complications of preterm birth (2013). Across 184 countries, the rate of preterm birth ranges from 5% to 18% of babies born. In India, out of 27 million babies born every year (2010 data), 3.5 million babies born are premature [4].

In many countries rates of premature births have increased between the 1990s and 2010s. Complications from preterm births resulted in 0.81 million deaths in 2015 down from 1.57 million in 1990. The chance of survival at fewer than 23 weeks is close to zero, while at 23 weeks it is 15%, 24 weeks 55% and 25 weeks about 80%. The chances of survival without long term difficulties are lower.

Pregnancy interval makes a difference as women with a six-month span or less between pregnancies have a two-fold increase in preterm birth. Studies on type of work and physical activity have given conflicting results, but it is opined that stressful conditions, hard labour, and long hours are probably linked to preterm birth [5, 6].

A history of spontaneous (i.e., miscarriage) or surgical abortion has been associated with a small increase in the risk of preterm birth, with an increased risk with increased number of abortions, although it is unclear whether the increase is caused by the abortion or by confounding risk factors (e.g., socioeconomic status). Increased risk has not been shown in women who terminated their pregnancies medically. Pregnancies that are unwanted or unintended are also a risk factor for preterm birth [1, 4, 7].

Aetiological and Risk Factors Associated with preterm:

There are several risk factors associated with preterm deliveries such as Behavioural and socio-demographic factors: it includes sociological, economical, racial, age marital status, educational etc, then medical factors such as previous or familial history of PTD, it is further classified into genetic factor as gene mutation or polymorphisms, environmental factors gases or UV radiation, biological hormonal pathways, idiopathic factors. In addition to that there are several other factors such as endocrinal and tissue remodelling factors where the biochemical or hormonal changes takes place [8].

Metabolically there are numerous pathways which directly involve in promoting successful pregnancy where else there is one of the most notable factors is folate pathway. If any defect in these pathway lead to pregnancy complication [9]. A hyperhomocystenemia a change of metabolisms had found to be a leading cause for preterm deliveries. Studies had proved that increased level of plasma homocysteine creates massive disturbance in genetically. Methylene tetrahydrofolate reductase (*MTHFR*) is the enzyme of methyl cycle which had been encoded by *MTHFR* gene. It promotes the catalysing of 5, 10 methylenetetrahydrofolate to methyl tetrahydrofolate, which acts as a co substrate for methylation to methionine. It initiates the genetic mutation of an enzyme which leads to *MTHFR* and coagulation protein Factor V. the enzyme of *MTHFR* is related to amino acid metabolisms. It leads to several genetic disorders such as intra uterine growth retardation , low birth weight, etc. along with it also affects the mothers health by increasing blood pressure, infection etc. [10].

2. MATERIALS & METHODS

2.1. Patient enrolment and stratification

For this study, mothers undergoing delivery (n=50) were selected, under the strict supervision of a registered medical practitioner along with concern of the patient. Pregnant women of the sub urban population undergoing preterm delivery (n =25) was selected on the bases of the gestation period (<37 weeks of gestation). Preterm delivery cases were further stratified into three cohorts in accordance to the gestational period as: extremely preterm (24-28 weeks), very preterm (28 to <32 weeks) and moderately or late pre-term (32 to <37). In addition to this full term and post term delivery cases (n = 25) were selected as control group, with all the clinical findings for both pre as well as term deliveries from MGM Medical College and Hospital, Kalamboli, Navi Mumbai. Pregnant women with Jaundice, Tuberculosis, HPV infection, HBV, urinary tract infection and miscarriage, pregnant female's twin foetus, foetus with congenital anomalies, IUGR had been excluded.

2.2. PCR-RFLP analysis of *MTHFR* C677T polymorphism

Peripheral blood was collected from MGM Hospitals in EDTA vacutainers and was further processed with DNA isolation. DNA extracted from peripheral blood using a HiPurA™ Blood Genomic DNA Miniprep Purification Kit. The genes were genotyped using the polymerase chain reaction–restriction fragment length polymorphism method. Genotypically the genes were analysed with the help of polymerase chain reaction–restriction fragment length polymorphism method (PCR RFLP) technique. The technique was first used for identifying 677C→T [10]. Using primer of 198 bp long the genes were amplified 5' TGAAGGAGAAGGTGTCTGCGGGA3' and 5'AGGACGGTGCGGTGAGAGTG3', later by using *HinfI* restriction and digestion was processed. For codon 677 wild type C allele represents a single band of 198 bp. Where else 198, 175 and 23 (heterozygous) and 175 and 23 (homozygous) represents the presence of T allele codon 677 of *MTHFR* gene.

3. RESULTS & DISCUSSION

Deliveries occurring before 37 weeks of gestation are termed as preterm. For the present study, 25 pregnancy cases were studied on the basis of gestation weeks as term delivery cases, extremely preterm (24-28 weeks), very preterm (28 to <32 weeks) and moderately or late pre-term (32 to <37) [2, 11, 12]. The maximum count of the enrolled preterm cases were of the moderate or late preterm (60%), where else the least count of preterm cases were of extremely preterm (12%), another study resembles same findings were seen as the moderate or late preterm (68.90%), where the least number of preterm cases was identified in the extremely preterm (10.53%) and babies born between 32 and 37 weeks of gestation moderately preterm (n=80%) [9]. Another study even showed similar result as babies are born between 28 and 32 week (10%) and only 5% of babies are born before 28 week of pregnancy [11].

As from the previous literature we found that polymorphisms of *MTHFR* C677T have associated with pre term delivery. The current study, we tried to analyze the polymorphisms of *MTHFR* gene

and its association in preterm labour in Navi Mumbai population by the gene product and was analysed by PCR- RFLP technique using *HinfI* restriction digestion. The PCR amplification of *MTHFR* showed the 198 bp band present (Fig 1). But after restriction and digestion only fragments of 198bp (wild type) were observed for all 25 samples, no results were shown for three bands of 198 + 175 + 23 bp (heterozygote) or two bands of 175 + 23 bp (homozygote) as a RFLP product after restriction digestion (Fig 2). Thus, it could be due to very less number of samples to detect the polymorphism.

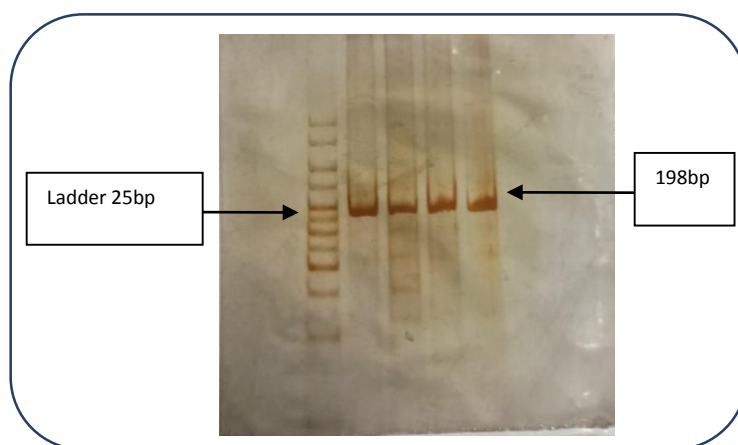


Figure 1: Representation of polyacrylamide gel electrophoresis for PCR amplification of *MTHFR* gene, representing the presence of wild type 198bp.

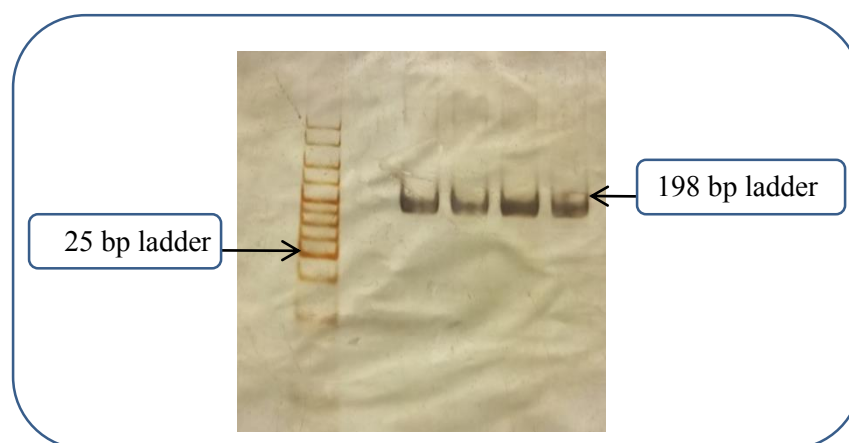


Figure 2: Representation of polyacrylamide gel electrophoresis for PCR RFLP of *MTHFR* gene, representing the presence of only wild type 198 bp and no polymorphisms seen.

Decrease in the level of folate intake had been proved to promote decidual vasculopathy and end up in preterm birth [13].

We would conclude that, in our study the polymorphisms of *MTHFR* 677C→T were not found. It could be due to less number of samples, as the other studies had more number of sample size, though they could identify only 2% polymorphisms of *MTHFR* C677T gene. To conclude, the present study requires more number of clinical samples, which would then effectively highlight

the association of genetic factors in preterm deliveries and its related complications.

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5. CONFLICT OF INTEREST

Authors report no conflict of interest is involved in this work.

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Efficacy of New Anti-Anaemic Preparations Using Biologically Synthesized Iron Nanoparticles in Wistar Rats

S. KULKARNI¹, N. MOHANTY², N. KADAM², N. SWAIN³, R. PATIL¹, M. THAKUR^{1*}

¹Department of Medical Biotechnology, MGMSBS, MGMIHS & MGM CET, Navi Mumbai, India

²Department of Pediatrics, MGM Medical College, MGMIHS, India

³Department of Oral Pathology, MGM Dental College, MGMIHS, India

ABSTRACT: A very common nutritional disorder “Iron Deficiency Anaemia” is a global public health problem. Ferrous sulfate is the most commonly used supplement for treating anaemia, but it can result in unfavourable side effects. Nowadays, nanotechnology is used as a way to increase bioavailability and decrease the side effects of drugs and nutrients. This research work introduces biologically synthesized nanoparticles (IONPs) as a new modality for treatment of IDA. This study investigates the efficacy of biologically synthesized IONPs at different doses in comparison to ferrous sulfate in anaemic rats. 10-50 nm nanoparticles were synthesized and characterized for its physicochemical, biological and toxicological properties. Iron deficient anaemia was induced by using iron deficient feed given orally to rats for 42 days. Wistar albino female rats were randomly divided into 6 groups - Group 1 – Control, Group 2 – Vehicle control, Group 3 – IDA without treatment, Group 4 – IDA with 1 mg/kg IONPs dose, Group 5 – IDA with 5 mg/kg IONPs dose and Group 6 – IDA with FeSO₄. Within only 5 days, administration of a single dose increases haemoglobin concentration (Hb) from 6.9 g/dL to 15.2 g/dL and RBCs counts from $4.7 \times 10^6/\text{mm}^3$ up till $6.6 \times 10^6/\text{mm}^3$ for Group 5. Moreover, the histopathological examination gives strong evidence that the used IONPs stimulate the erythropoiesis process without any apparent toxicity. The results revealed that Group 4 rats took 20 days and Group 6 rats took 28 days to recover from IDA by increase in haemoglobin levels. From this study, it can be concluded that 5 mg/kg dose of IONPs can be used as a new drug treatment of IDA.

KEYWORDS: Anaemia, ferrous sulfate, biologically synthesis, iron oxide nanoparticles, in vivo

***Corresponding Author: Dr. Mansee Thakur**

Department of Medical Biotechnology, MGMSBS, MGMIHS & MGM CET, Navi Mumbai, India

E-mail Address: mansibiotech79@gmail.com, mansimgm07@gmail.com

1. INTRODUCTION

Nanotechnology refers to a fastest growing scientific field with wide range of technologies that measure, manipulate, or incorporate materials and/or features with at least one dimension between approximately 1 and 100 nm. Progress in nanotechnology has brought countless novel applications in different areas such as material science, energy, or health [1]. Even though there are a number of publications available focussing on the undesirable side effects of nanotechnology, still there is a need of development in the health and safety aspects of nanotechnology. One example is given by iron oxide nanoparticles (IONP), which are finding a rapidly increasing number of biomedical

applications because of their suitable structural, colloidal, and magnetic properties [2]. Nowadays, IONPs are used in various platforms for bio sensing [3], bimolecular-magnetic trapping [4], hyperthermia [5], imaging [6], and drug or gene-delivery [7, 8].

As we know Iron is one of the most important trace elements in human body and is essential for the normal function of organisms, particularly for the metabolism and immune functions [9]. As reported by World Health Organization, 2 billion people have anaemia, with nearly 1 billion suffering from iron deficiency anaemia (IDA) [10]. IDA is therefore a common nutritional disease in modern society [11]. Ferrous salts, is common in the treatment and prevention of iron deficiency in human body as an oral iron supplements [12]. However, it usually causes side effects, such as epigastria pain, diarrhoea and constipation [13]. Therefore, it is highly desired to develop new iron supplements with no or few side effects [14].

With the novel properties of nanoparticles, new opportunities have evolved for the drug delivery and treatment of various diseases. There are literatures available focusing on gastrointestinal absorption of nanoparticles on enhancing absorption of drugs, vaccines and nutrients that either are degraded by the digestive process or poorly absorbed. Hence, several iron nanoparticles were synthesized to treat IDA with great efficiency and low side effects instead of the frequently used iron salt [15, 16]. The objective of our study was to biologically synthesize iron oxide nanoparticles and evaluate their potential as an iron supplement in the treatment of IDA compared to a commercially available iron supplement (FeSO_4).

2. MATERIALS & METHOD

All the chemicals used were of analytical grade and purchased from Hi-Media.

2.1. Biosynthesis and Physico-chemical Characterization of Iron Oxide Nanoparticles (IONPs)

Iron oxide nanoparticles were biologically synthesized by hydroponically grown *Spinacia oleracea* plant leaf extract. These nanoparticles were further investigated for its size, morphology, state and Fe content using various sophisticated instruments including Scanning Electron Microscope (SEM), Energy Dispersive Spectroscopy (EDS), Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD) and Atomic Absorption Spectroscopy (AAS) [17].

2.2. Experimental groups

30 Wistar albino female rats were maintained in animal house of MGMIHS 2-3 week prior the study. The rats were randomly divided into six different groups (Figure 1). The iron deficient diet was purchased from VRK Nutritional Solutions, Pune. Before starting the study the blood was withdrawn from rats of each group and haematological indices were analysed. Group 1 & 2 were fed with normal diet whereas treated groups (Groups 3, 4, 5 & 6) were fed with iron deficient diet for 42 days.

2.3. Clinical haematological variables & Histopathological Analysis

Blood was collected by retro-orbital in heparinized tubes and level of haemoglobin (Hb), platelets (PLT), red blood cell (RBC) count and white blood cell (WBC) count were measured using a Sysmex haematology analyzer (KX - 21). The liver of rats from each group were dissected and then put in 10% formalin for further histopathology examinations.

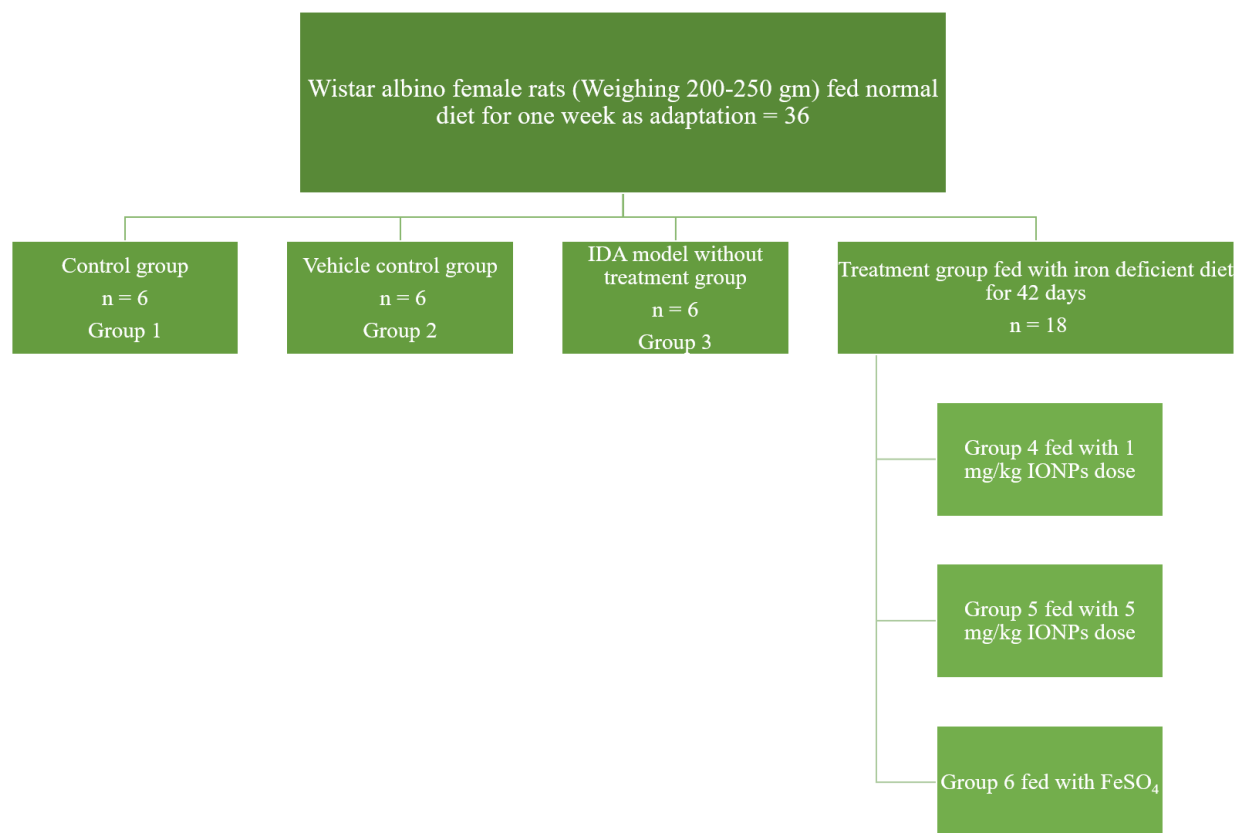


Figure 1: Experimental Design: Groups of Wistar Rats Involved in the Study

2.4. Statistical Analysis

The data are represented as mean $\pm$ SE. The significant differences were observed by t test. $P < 0.05$ was considered as significant.

3. RESULTS AND DISCUSSIONS

Anaemia is considered to be one of the most predominant nutritional problems in recent years [18]. The present study aims in developing biologically synthesized IONPs from herbal extract of *Spinacia oleracea* and if these nanoparticles could act as supplement in IDA Wistar rats. The physico-chemical characterization of biosynthesized IONPs revealed with spherical shape and average size of 10-50 nm particles that have been confirmed by SEM analysis (Figure 2). In vivo toxicity and possible side effects of nanomedicines have to be carefully studied before practical applications in clinic.

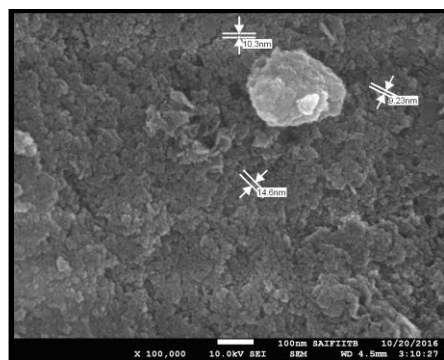


Figure 2: SEM Analysis of Biosynthesized IONPs revealing 10-50 nm size with Spherical Shaped Particles

To study the effect of the IONPs on IDA, low-iron diet wistar rats were separated into different groups and fed with the designed dosage of 1.0 mg/kg and 5.0 mg/kg IONPs and FeSO₄. The rats were weighted every week. The haematological indices are important parameters for the diagnosis and therapy of IDA. Therefore, the blood analyses were carried out for the rats studied and the results were presented in table 1.

Results presented in table 1 indicated that the Group 1 & 2 shown having normal values during experiment. Whereas group 3 exposed with IDA feed showed gradual decrease in Hb level throughout most of the follow-up weeks and were sacrificed at 7 weeks without showing improvement in iron status. Rats in Groups 4, 5 and 6 reached normal Hb and Hct values. All treatment groups (group 4, 5 and 6) showed significant ($p=0.05$) increase in Hb concentrations in both two doses (1 mg/kg and 5 mg/kg exposed) along with FeSO₄ duration days with dose relative to the mean baseline blood Hb after induction of anaemia as shown in table 1. All groups exceeded normal values at different speeds or durations. Similar results have been previously reported [19]. The higher efficacy of iron oxide nanoparticles relative to commercial iron supplement product can be explained by the enhanced bioavailability and therapeutic effectiveness of iron by increasing the surface area.

Hematological Indices of Wistar Rat Blood									
	Group 1	Group 2	Group 3	Group 4		Group 5		Group 6	
	Normal Feed	Normal Feed	After IDA Feed	After IDA Feed	After Treatment with IONPs dose of 1 mg/kg (20 days)	After IDA Feed	After Treatment with IONPs dose of 5 mg/kg (5 days)	After IDA Feed	After Treatment with FeSO ₄ (28 days)
WBC*10 ³ /μL	16 ± 0.4	15.9 ± 0.5	14.3 ± 0.4	14 ± 0.4	15.2 ± 0.9	14.3 ± 0.5	15.7 ± 0.5	14.1 ± 0.5	15.3 ± 1.2
RBC *10 ⁶ /nL	6.5 ± 0.1	6.6 ± 0.1	4.6 ± 0.3	4.6 ± 0.3	6.5 ± 0.2	4.7 ± 0.2	6.6 ± 0.2	4.6 ± 0.4	6.6 ± 0.2
HGB (g/dL)	14.3 ± 0.6	14.8 ± 0.9	7.7 ± 0.2	7.6 ± 0.4	14.4 ± 0.9	7.3 ± 0.4	15.2 ± 0.8	7.1 ± 0.4	14.7 ± 0.5
HCT (%)	41.5 ± 0.8	41.7 ± 0.8	18.3 ± 0.4	18.3 ± 0.3	41.6 ± 0.9	18.5 ± 0.2	42.1 ± 1.2	18.5 ± 0.2	42.1 ± 0.5
MCV (fL)	64.6 ± 1.3	64.9 ± 1.1	46.1 ± 2.2	46.4 ± 2.1	64.4 ± 1.3	46.9 ± 1.6	64.8 ± 1.1	45 ± 2.4	64.2 ± 2.3
MCH (pg)	19.4 ± 0.4	19.3 ± 0.5	15.8 ± 0.2	15.5 ± 0.4	19.3 ± 0.4	15.6 ± 0.4	19.4 ± 0.4	15.8 ± 0.4	19.3 ± 0.6
MCHC (g/dL)	30.3 ± 0.5	30.3 ± 0.5	21.3 ± 0.2	21.3 ± 0.7	29.9 ± 0.5	20.7 ± 0.6	30.1 ± 0.6	20.8 ± 0.6	30.1 ± 0.6
PLT *10 ³ /nL	989.5 ± 23.4	991.8 ± 18.4	1377.2 ± 22.4	1366.7 ± 31.3	1010.2 ± 36.3	1377.2 ± 34	1004.8 ± 39.2	1374.7 ± 28.5	1003.7 ± 31.3

Table 1: Haematological Indices of Wistar Albino Female Rats after Inducing IDA and After Treatment with IONPs and FeSO₄

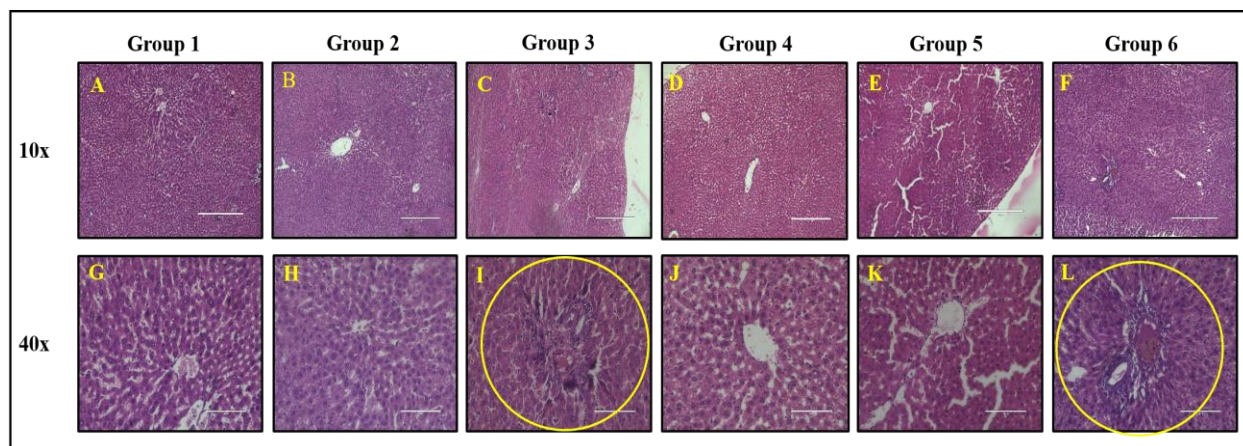


Figure 3: Histopathological Analysis of Wistar Albino Rat Liver

Microscopic examination of H & E stained liver tissue of Group 3 (IDA model without treatment) revealed additional histological changes like portal infiltration with mononuclear inflammatory cell infiltration, nuclear pyknosis and atypia in hepatocytes (Figure C & I) as compared with the observations of control - group 1 (Figure A & G) and vehicle control – group 2 (Figure B & H). On comparison of the observations of the treatment groups, only the specimens treated with FeSO₄ showed congestion of portal vein along with lymphocytic infiltration and mild nuclear atypia where as other treatment groups did not show significant changes. To summarize the microscopic changes of all groups, it can be concluded as the histopathological changes observed in samples with IDA had seen to be improved in groups treated with IONPs (Group 4 & 5) rather than FeSO₄ (Group 6) [19, 20, 21].

Our study also shown similar pattern what Singh [22] who illustrated that generally the internalization of NPs within cells is likely to occur in a time-dependent manner followed by plateau when cells reach maximum saturation. Similarly, Fischer et al. [23] reported that cell viability subsequent to nanoparticle uptake is expected to be either unaffected or decreased as a function of time. Recent researchers have demonstrated that nanonization of poorly absorbed drugs can improve cellular uptake and oral bioavailability of these drugs [24, 25]. All results demonstrate the high therapeutic effect of the IONPs on IDA in vivo. As a result, the IONPs are highly promising as an IDA drug.

4. CONCLUSION

We have designed and experimentally demonstrated the IONPs for the targeting IDA treatment. In vivo toxicity studies were carried out using the oral administration of animal model. The blood analysis showed that the IONPs have potent therapeutic effect on IDA, evidenced by the HGB, RBC and HCT values of or both the doses at 1mg/kg/d and 5.0 mg/kg /d IONPs treated IDA rats. For early relief the IONPs at 5mg/kg for 5 days are promising candidates for IDA drugs.

5. ACKNOWLEDGMENT

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6. CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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Understanding Correlation between Moderate Anemia & Hemolytic Events in Sickle Cell Disease: A Study from Tribal Rural Western Maharashtra

N. N. SATAM¹, V.W. PATIL², T. MARAR¹, D. GARG^{1*}

¹School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

²Department of Biochemistry, Grant Government Medical College and Sir J. J. Groups of Hospital, Mumbai, Maharashtra, India.

ABSTRACT: Sick cell disease (SCD) is the most common inherited monogenic disorder in Indian tribal and non-tribal population SCD pathogenesis is widely studied at National and International levels, most important of these are pain episodes and vaso-occlusive crisis. In the present study, we investigate the frequency of hemolytic events in SCD and its correlation with moderate anemia in tribal and rural population from Palghar, Western Maharashtra. Sick cell subjects (n=250) as tests & age and sex matched healthy persons (n=250) as controls, from primary health centers of Palghar were included in this study. Informed written consent was obtained from the all subjects who were enrolled. They were screened and conformed for SCD by solubility test and HPLC and a complete blood count was conducted for all subjects. We observed that anemia was observed significantly more often in patients with sickle cell disease compared with sickle cell trait & control ($p < 0.001$) of all ages. Moderate and mild anemic condition was also present in control subjects. The RBC indices and morphology of presented by sickle cell disease (SS) subjects were significantly altered as compared to sickle cell trait (AS) and controls (AA). Comparison among hemolytic events versus vaso-occlusive single events suggests that, hemolytic events, pallor and yellow sclera counted more than other single vaso-occlusive event. Further in depth study is necessary for proper understanding of pathogenesis of sickle cell disease.

KEYWORDS: Sick cell disease, anemia, hemolytic events, vaso-occlusive crisis

***Corresponding Author: Dr. Deepa Garg**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: deepa.garg@dypatil.edu

1. INTRODUCTION

Sickle cell disease (SCD) is molecular basis of the prototypic genetic disorder, present due to substitution of the valine in the position of glutamic acid at 6th position of the hemoglobin beta chain on chromosome [1]. This structural variation results in reduced solubility of the mutant gene product Hemoglobin S (HbS). Homozygous sickle cell disease (SS) is a result of dominant inheritance from both parents, whereas sickle cell trait or heterozygous (AS) result from inheritance of sickle gene from one parents (S) & normal gene (A) from other parents [2]. Indian hypotype is believed to be milder due to alpha thalassemia & fetal hemoglobin (Hb F) in tribes. However, severity of illness needs to be assessed based on socioeconomic, environmental &

nutritional status of tribal and non-tribal population group. Nutritional anemia is highly abundant in India. It is also reported that low iron content in RBC protects normal individual from the pathogenesis of malaria [3]. It has been reported that excess iron loss is due to increased urination leading to iron deficiency in adult SCD patients and sensitivity of iron detection test by means of serum ferritin is low in SCD cases and hence it's not easy to correctly identify iron deficiency with this method.

It is also advised not to supplement iron to SCD patients if they are pregnant [4]. Kinetics of sickling is high in subjects with high MCHC. Small decrease in MCHC in SCD causes substantial delay in Hb S polymerization. Delay in HbS polymerization helps RBC faster transit time in the circulation with delay in gelation helps to avoid sickling process.

In this study theoretical aspects of two important types of pathogenesis were considered for differentiation of SCD phenotypes. In hemolysis type, when the polymerized hemoglobin S attached to each other forms straight rods of 6 to 14 stranded structures, the cell gets deformed and most of the free heme is transported to the inner membrane of the red cell. This heme may be toxic to the membrane and that may lead to lyses of cell membrane. Pain episodes are the final consequences of vaso occlusive crisis. Vaso occlusive crisis is mainly due to clogging of the venules which in turn resulting from inflammating pathways i.e. due to modified membrane of blood cells, which includes cytoadhesion of RBC, WBC & platelets to endothelial cells. In the present study clinical events of SCD patients categorized in to two phenotypes [5] i.e. vaso-occlusive vs hemolytic crisis were analysed in the background information on Hb levels in the community as per WHO grading system to correlate inherent nutritional anemia the community with clinical events in SCD patients.

2. MATERIALS AND METHODS

SCD diagnosis was carried out in different location in Palghar district from western India. Conformation of SCD was done through solubility test [6, 7] and HPLC method [8]. RBC morphology study was conducted by auto-analyzer. Family studies by pedigree analysis from all available data were also carried out for final confirmation of sickle cell homozygous patients and sickle cell heterozygous carrier. We have followed the ethical guidelines of 1975 declaration of Helsinki and informed consent was taken from adult population. IEC clearance was obtained from GGMC and Sir J.J.groups of hospitals, Byculla, Mumbai.

3. RESULT AND DISCUSSION

Clinical events of SCD cases were categorized into hemolytic crisis and vaso occlusive crisis. Cross section data of hemoglobin levels of all carriers & normal population were categorized as per WHO grading system for anemia. Inherent moderate anemia in the community was correlated with clinical events of SCD cases to assess the possible contribution of inherent moderate anemia.(Table 2&3) In this random population study total male were 238 average age were 14.42 years & total female were 262 average age were 15.23 years. From total number of subjects studied N=500, 250 were control (AA), 201 were sickle cell trait (AS), 49 were Sickle cell disease (SS).

Maximum no. of subject from our studies was of Schedule tribes i.e. Warli, Kakari, Malharkoli, Dhorkoli, Vanjari, Wadwal. Schedule caste population comprised of Buddhist followed by Mahar. Previous study of Kate SL [9], Sukla RM [10], Patra et al [11] and many more also reported that SCD was prevalent in Schedule tribes, Schedule caste, few in other backward castes & very rare in general categorizes.

Hemoglobin % (Hb %) (Table 1) was significantly decreased in SCD subjects but sickle trait had Hb% compared to control. We categorized this hemoglobin level according to WHO guidelines for anemia identification. Walke et al [12] also reported mean Hb% was 10.8% in sickle cell trait in pediatric population. But they did not further classify it further based on anemic condition. In our observations, there was direct correlation between Hb% in subjects & the clinical features. Subjects with SCD reported a history of weakness, breathlessness associated low Hb%.

RBC count (Table 1) of SCD subjects was significantly lower than that of traits & control. When study by Pathak et al [13] compared with our study, they observed mean RBC count in AS (Trait) was statistically significant. Mean RBC count in SCD was found to be 2.97 million/cmm in our study, which was lower than that of study by Yasmin et al [14]. They have reported mean RBC count in AS subject was 3.87 million/cmm.

Hematocrit (HCT) (Table 1) value in SCD subjects also decreased significantly. In the reports of Walke & Walde [12], HCT values between trait and control was not significant as seen in our study also. Mean MCV (Table 1) value in our study was decreased in SCD subjects & found statistically significant. Values of control & sickle trait subjects are almost equal. Patel et al [15] reported mean MCV, which was similar to our mean MCV in SCD subjects. But in study of Yasmin et al [14] mean MCV was in normal range for sickle cell trait. Mean MCH (Table 1) value in SCD subject was also found significantly altered. Similar results were reported by Pathak et al [13]. The value reported by Yasmin et al [14] was similar to our study. We observed significant difference in SCD subjects in the value of MCHC. This has not been reported by any studies. Cross section clinical findings (Fig.1) suggested that hemolytic events, pallor (35 SCD cases) & yellow sclera (31 SCD cases) were found to be more than any single clinical even related to vaso occlusion based pathogenesis which were noted as joint pain (24 SCD cases), Chest pain (12 SCD cases), abdominal pain (10 SCD cases), pain episodes (13 SCD cases), palpable spleen (7 SCD cases), palpable liver (2 SCD cases), ulcer manifestation (8 SCD cases). As assessment by ANOVA with value of $p < 0.001$ was consider statistically significant for the analysis of Hb levels for different age groups (Table 2, 3) as well as genotypes.

4. CONCLUSION

Population survey of hemoglobin levels in control & SCD subjects indicates that moderate anemia is most abundant in this geographic location & in all studied variables in homozygous SCD cases, frequency of hemolysis based clinical events i.e. pallor & yellow sclera were found to be more that of any other vaso- occlusion based single clinical event. Further need of biochemical analysis of RBCs as well as molecular insight in this studied population required to understand correct pathogenesis.

Parameters	Control(AA)	AS	SS
Hb g/%	10.67 + 0.06	10.26 + 0.08	7.75 + 0.11*
Hematocrit%	28.24 ± 0.22	27.15 ± 0.26	19.76 ± 0.15*
TCL(x10⁹L⁻¹)	6.33 ± 0.8	7.94 ± 0.20	10.75 ± 0.35*
RBC(x10¹²L⁻¹)	4.04 ± 0.03	4.15 ± 0.02	2.97 ± 0.03*
Platelets(x10⁹L⁻¹)	369.56 ± 3.5	371.51 ± 3.94	214.93 ± 6.07*
MCV (fL)	79.80 ± 0.14	78.94 ± 0.18	73.56 ± 0.34*
MCH(pg)	30.38 ± 1.08	30.36 ± 1.36	25.70 ± 0.24*
MCHC(g/dL)	38.31 ± 0.15	37.6 ± 0.18	32.07 ± 0.22*

Table 1: RBC morphology studies in control and Subjects population
Values are expressed as Mean ± SE and significance is at *P <0.001

Distribution of Hb Levels in males with or without Hemoglobinopathies			
Hb (g/dl)	AA	AS	SS
<7	0	0	5
7.1-9.9	13	41	27
10-12.9	89	48	0
≥13	12	3	0
Total	114	92	32

Table 2: Levels of anemia as per WHO grading system in males

Distribution of Hb Levels in Females with or without Hemoglobinopathies			
Hb (g/dl)	AA	AS	SS
<7	0	0	3
7.1-9.9	39	44	14
10-12.9	87	65	0
≥13	10	0	0
Total	136	109	17

Table 3: Levels of anemia as per WHO grading system in females

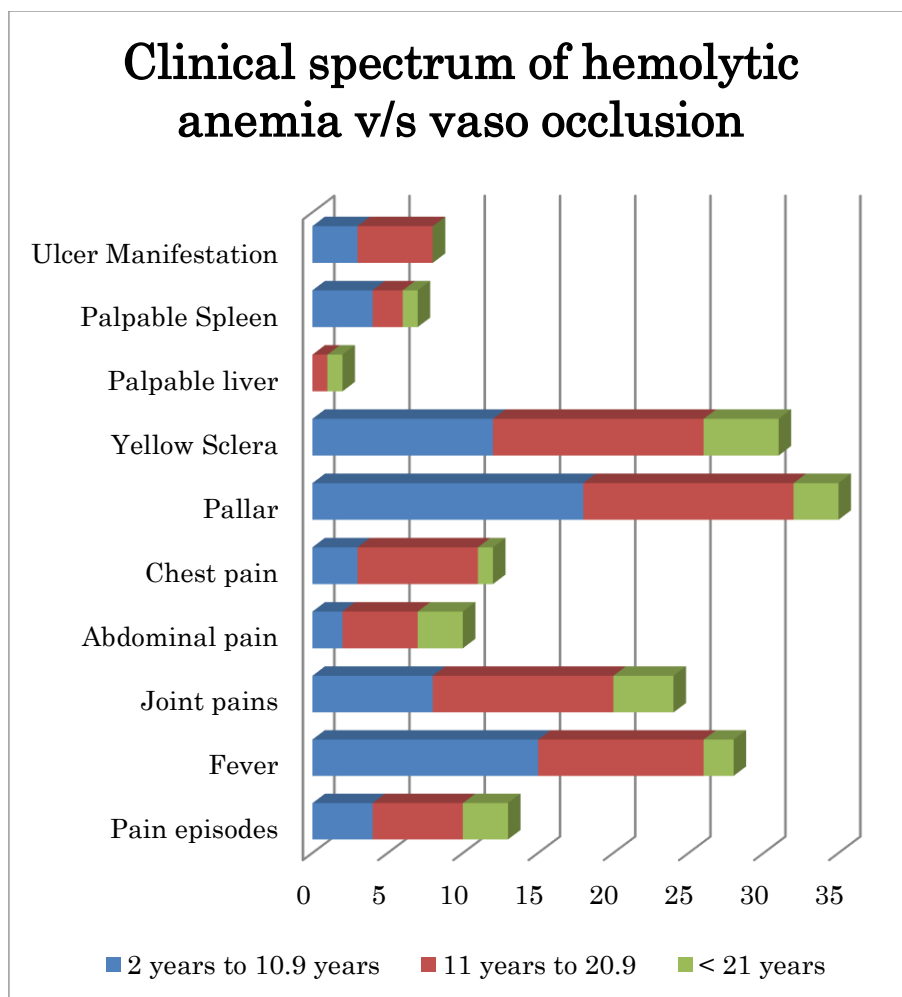


Figure 1: Clinical spectrum categorised in two phenotypes. Number of subjects are mention on X axis and types of events on Y axis.

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Study of Drug Resistance Organisms from Fomites

J. SHAH, M. AGARWAL, S. GORE, S. RATHOD*

Department of Microbiology, K.C. College, Mumbai-20

ABSTRACT: Fomites are inanimate objects that can serve as vehicle for pathogen transfer. Antibiotic resistance of micro-organisms is one of the grievous challenges of the 21st century. The life-saving drugs have ceased to work because of the increasing emergence of microbial strains resistant to them. The aim of this study is to establish the possible presence of antibiotic resistant bacteria on fomites in the trains (western railway) and evaluate their antibiotic susceptibility pattern. 48 samples from various areas of train compartments were used for the study. Out of which 14 isolates were identified using conventional VITEK method. Antibiotic susceptibility testing of all the isolates was performed using Disc diffusion method. A variety of organisms were isolated including a few pathogens. Most of the isolated organisms were found to be resistant to multiple antibiotics. The effect of essential oils like cinnamon and clove bud was tested on the isolated organisms and was found to inhibit them at a concentration of 0.25 to 1%. To prevent the spread of such resistant organisms proper sanitation and hygiene awareness has to be brought about as they pose a threat to public health.

KEYWORDS: Antibiotic Resistant organisms, antibiotic susceptibility, fomites, train, essential oils

***Corresponding Author: Ms. Sejal Rathod**

Department of Microbiology, K.C. College, Mumbai-20.

Email Address: sejal.rathod@kccollege.edu.in

1. INTRODUCTION

Fomites consists of either porous or non-porous surfaces that when contaminated can act as a vehicle for transmission of pathogens. Fomites can be contaminated with aerosolized bacteria, contact with fluids etc. Many common places are shared by people increasing the risk of transmission and infection. Public transport can lead to bacteriological, viral or fungal infections [1]. Contributing to the complexity is an incomplete understanding of indirect contact transmission. Indirect contact transmission refers to person-to-person transmission of disease via an intermediate fomite (i.e., inanimate object acting as a carrier of infectious disease). Indirect contact, or fomite-mediated contact, is poorly understood due, to the nature of the transmission route. There are a number of ways fomites can be contaminated with infectious disease, including contact with bodily fluids, body parts, or other fomites and settling from airborne particles by talking, sneezing, coughing, or vomiting [2]. Contamination of a fomite may provide no obvious or visible evidence of infectious disease presence. Additionally, the routes by which an infectious agent contaminates a fomite are equally able to infect a susceptible individual without the intermediate fomite. Therefore, it is often difficult to determine whether a transmission event occurred directly between an infected host and a susceptible host, or the event occurred indirectly

via a fomite. This study focuses on the detection of drug resistant organisms.

2. MATERIALS AND METHODS

2.1.Collection of samples

Samples were collected from different parts of train (handle, rod, seat and window) by swab method [3].

2.2.Isolation

Isolation was carried out on sterile Mac Conkey (selective) and Nutrient agar (Hi - Media) [4].

2.3.Identification of Bacterial Isolates– Cultural and Morphological Characteristics

Sub-culturing was carried out on distinct colonies until pure cultures were obtained and were preserved. Individual colonies were purified and identified by morphological and biochemical techniques and further identified by biochemical examination and VITEK method.

2.4.Antibiotic Susceptibility Test

Antibiotic Susceptibility testing was performed by Kirby-Bauer test also called as Disc Diffusion test on sterile Muller-Hinton's media (Hi - media) and using standard procedure of the Clinical and Laboratory Standards Institute. Antibiotic discs of standard concentrations were placed on agar plates swabbed with isolated organisms and the susceptibilities of the organisms to the antibiotics were determined. The antibiotics used were as follows: Ampicillin (AMP¹⁰), Amoxycylav (AMC³⁰), Cefotaxime (CTX³⁰), Chloramphenicol (C³⁰), Ciprofloxacin (CIP⁵), Gentamicin (GEN¹⁰), Tetracycline (TE¹⁰) and Ticarcillin (TI⁷⁵) [4,5].

2.5.Determination of Minimum Inhibitory Concentration

Antimicrobial activity of herbal essential oils cinnamon (*Cinnamomum zeylanicum*) and clove bud (*Syzygium aromaticum*) was tested by Agar dilution method. The suspensions of the 14 isolated cultures were adjusted to a turbidity of 0.5 McFarland. The Nutrient agar was used in order to obtain concentrations from 50µl (0.25%) to 200µl (1%) and plates were poured. Saline suspensions of the 14 isolates were spot inoculated on to the plates. The lowest concentration was capable of inhibiting visible growth after 24 hours of incubation at 37°C was then recorded as the MIC [4, 6, 7].

2.6.Formulation of the herbal disinfectant spray was done.

3. RESULTS AND DISCUSSION:

3.1.A total of 48 samples were collected from trains at peak timings.

3.2.13 Gram negative and 1 Gram positive isolates were selected.

3.3.Identification revealed a variety of micro-organisms, most of them were opportunistic pathogens.

3.4.The minimum inhibitory concentration (MIC) of cinnamon and clove bud was found to be 0.46% (92µl) and 0.25% (50µl) respectively.

SR. NO.	ISOLATES	ORGANISMS	SITES
1	4	<i>Enterobacter cloacae complex</i>	Shelf
2	5	<i>Enterobacter cloacae complex</i>	Window
3	6	<i>Pantoeaspp</i>	Seat
4	9	<i>Enterobacter cloacae complex</i>	Seat
5	10	<i>Rhizobium radiobacter</i>	Handle
6	11	<i>Cronobacter sakazakii group</i>	Shelf
7	12	<i>Acinetobacter baumannii complex</i>	Seat
8	13	<i>Enterobacter cloacae complex</i>	Window
9	14	<i>Pseudomonas stutzeri</i>	Window
10	16	<i>Kocuria rosea</i>	Shelf
11	17	<i>Alcaligenes faecalisspp</i>	Seat
12	18	<i>Pseudomonas stutzeri</i>	Window
13	20	<i>Acinetobacter lwoffii</i>	Shelf
14	21	<i>Acinetobacter baumannii complex</i>	Window

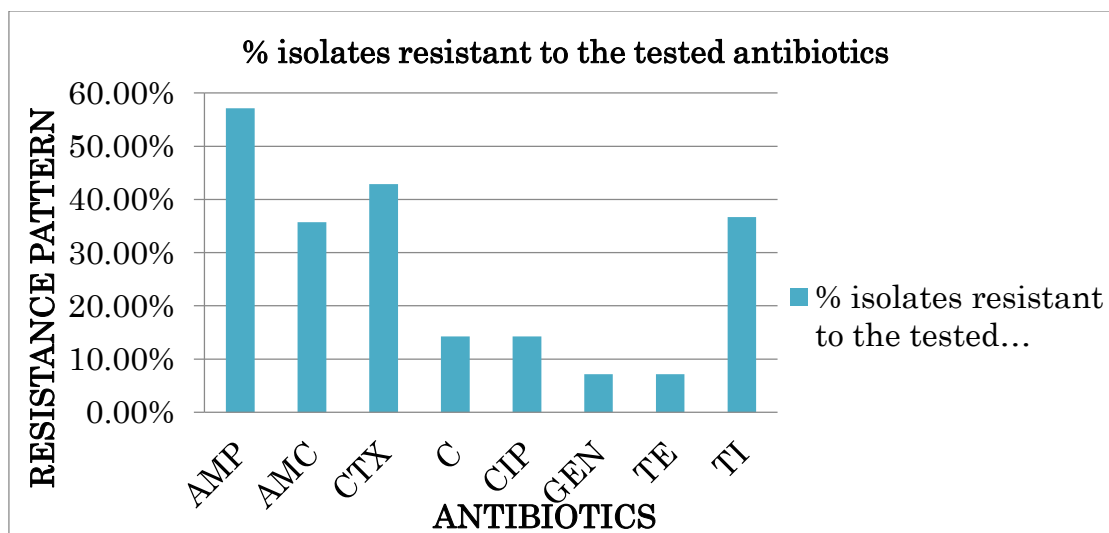
Table 3.1: Identification of isolates by Vitek method

SR. NO.	ISOLATES	RESISTANCE PATTERN
1	4	AMC
2	5	AMP , AMC
3	6	AMP, CTX, TI
4	9	TE, TI
5	10	TI
6	11	AMP
7	12	AMP , AMC, CTX, C, TI
8	13	AMC
9	14	-
10	16	AMP, CTX, CIP
11	17	AMP, CTX, C, TI
12	18	GEN
13	20	CTX, CIP
14	21	AMP , AMC, CTX

Table 3.2: Antibiotic resistance pattern of bacterial isolates from trains.

KEY- Ampicillin (AMP¹⁰), Amoxyclav (AMC³⁰), Cefotaxime (CTX³⁰), Chloramphenicol (C³⁰), Ciprofloxacin (CIP⁵), Gentamicin (GEN¹⁰), Tetracycline (TE¹⁰) and Ticarcillin (TI⁷⁵).

Multi drug resistant organisms: - Isolates: 6, 12, 16, 17, 21.



Graph 1: Percentage of isolates resistant to the tested antibiotics.

KEY-

ANTIBIOTICS	RESISTANCE (%)
Ampicillin(AMP ¹⁰)	57.14%
Amoxyclav(AMC ³⁰)	35.71%
Cefotaxime(CTX ³⁰)	42.86%
Chloramphenicol(C ³⁰)	14.28%
Ciprofloxacin(CIP ⁵)	14.28%
Gentamicin(GEN ¹⁰)	7.14%
Tetracycline(TE ¹⁰)	7.14%
Ticarcillin(TI ⁷⁵)	35.71%

4. CONCLUSION

The aim of the research work was to detect the presence and determine the number of antibiotic resistant organisms from fomites (train). Even though the number of organisms found varied, it was observed that there were certain organisms which showed varied resistance to antibiotics, some of them being MDR. These results indicate that spread of antibiotic resistance is alarming and indicate that antimicrobial agents should be better managed in order to reduce the emergence of resistance of organisms. There is a risk of commuters being exposed and infected by the resistant organisms.

Also there is a possibility of normal flora acquiring resistance. These organisms were found to easily infect immuno-compromised hosts. Better awareness needs to be generated among people and workers regarding proper cleaning and sanitization since thousands of people travel regularly by trains. Also awareness needs to be created upon the judicious use of antibiotics. Health of public is important and hence trains should be disinfected on a regular basis. After train travel it is very essential to sanitize your hands since transmission of pathogens can take place via contact too. Further work is needed to determine exactly where the resistance is originating from. To

devise a method to make the organisms susceptible to antibiotics they were resistant to. Appropriate care should be taken to avoid spread of organisms by following proper sanitization and cleaning methods.

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6. CONFLICT OF INTEREST

We have no conflict of interest that exists.

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Identification of Potent Flavonoids from Food against Over Activated MEK1 Target in Melanoma

S. DEVARAJAN^{1*}, V. ZAMBARE¹, D. SWAMI¹

¹School of Biotechnology and Bioinformatics, D. Y Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

ABSTRACT: The consumption of selected fruits and vegetables has been believed to reduce the risk of developing various types of human cancers. Flavonoids are the most common family of polyphenolic compounds with a lot of compounds identified so far. Many flavonoids found in vegetables, cereals and fruits, and beverages such as teas and coffees can be subdivided into 14 different categories. Flavonoids can reduce the growth of many different types of cancer cells through a variety of mechanisms. Several phytochemicals have been isolated, and a large number of them have shown positive effects in different disease types. However, there is not the much scientific evidence supports the protective effects of flavonoids from food against overactivated receptors in skin cancer type. The present study aims to identify the potential compounds from flavonols against the overactivated mitogen-activated protein kinase kinase 1 receptor (MEK1). A molecular interaction study was conducted between all the compounds with this receptor and further investigates the specificity, interaction and pharmacokinetic properties among them. After a GA based screening on the selected compounds, we have calculated the properties including LogP, Pgp efflux, and CYP metabolism. We identified a group of compounds which showed favorable molecular interaction in comparison with existing MEK1 drugs with respect to their molecular specificity. Our results revealed potent flavonoids from Isorhamnetin, Kaempferol, Quercetin and Spinacetin derivatives with promising binding affinity against the overactivated receptor MEK1 in skin cancer. However, Isorhamnetin 7-O-rhamnoside compound which is present in berry juices has to be focussed further based on the selectivity and property with the MEK1 receptor

KEYWORDS: Flavonoids, MEK1, Phytochemical, Isorhamnetin 7-O-rhamnoside, Skin cancer

***Corresponding author: Dr. Shine Devarajan**

School of Biotechnology and Bioinformatics, D. Y Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: shinedevraj@dypatil.edu

1. INTRODUCTION

Flavonoids are a group of natural chemical substances with polyphenolic structures and are found majorly in fruits, vegetables, tea, wine, roots, bark, stem, flowers, grains, etc [1, 2]. These natural compounds have various health benefits and are vital components of various medicinal preparations. Flavonoids have anti-mutagenic and anti-cancer properties [1, 2]. Flavonoids are classified into several classes like flavanols, flavanones, flavonols, isoflavones, flavones, and anthocyanins depending upon the variabilities in their structures [3]. Flavonols belong to a group

of colourless compounds called anthoxanthins which again belong to a major class, Flavonoids [4]. Skin Cancer or Melanoma is the most common neoplasm observed in the USA. Recently identified alterations in the function of specific genes in these cancers provide new potential therapeutic targets [5]. Over the past four decades, melanoma incidence has increased by nearly 200% [6]. Melanoma progresses by overactivation of mitogen-activated protein kinase kinase 1 (MEK1), an intermediate of the MAPK signaling pathway [7].

This pathway also known as RAS-RAF-MAPK signaling pathway, is overactivated in majority of the melanoma either by extrinsic growth factor signaling or by genetic alterations of intermediate protein members namely B-RAF and RAS[8-10]. Hence, targeting one of the intermediates becomes important for the treatment. Therefore, MEK1 can be tested as a potential molecular drug target against melanoma [11]. It has also been validated by *Zigang Dong et. al.* as a protein target [12] (PDB ID: 3EQH) Blocking the activity of MEK1 with flavonols prevents the activation of ERK, the successive protein in the pathway and hence stops the neoplastic cells from proliferating. Cereals and legumes, which is staple food in most of the developing countries, contain large amounts of polyphenols [13]. Hence, source is not a problem. Protein kinase inhibitors are a well-established class of clinically useful drugs, particularly for the treatment of cancer [14]. Dietary polyphenols have gained considerable attention for the prevention of UV-induced skin photodamage including the risk of skin cancer (Photocarcinogenesis) [15]. The SAR of flavonols states that the presence of C2-C3 double bond in the 'B' aromatic ring of the structure has an importance in the anticancer activity [16]. Almost all skin cancers can be treated successfully if diagnosed and treated early. However, if not treated, some types of skin cancer can be fatal [17]. Therefore, it is necessary to treat melanoma upon early diagnosis of cancer stage.

The present *in silico* study focuses on finding the best anticancer agent out of 76 available members of flavonol class by performing virtual screening. We have also calculated the pharmacokinetic properties of the top 10 compounds showed the best-docked poses in terms of the total energy and van der Waals forces.

2. MATERIALS AND METHODS

2.1.Target and library preparation

Structures of all the compounds in this study have been obtained from various online databases and many online servers. Open-source software has been used in this study. A brief overview has been given in the subsequent sections. PDB is the database for experimentally determined three-dimensional structural data of biological macromolecules such as proteins and nucleic acids. The structure of the human mitogen-activated protein kinase kinase 1 (MEK1) has been downloaded from the Protein Data Bank (PDB) PDB ID: 3EQH [18]. The structure has been visualized in Accelrys Discovery Studio Visualizer 3.5 in its single chain form in complex with the ligands.

Phenol-Explorer (<http://www.phenol-explorer.eu>) is the first comprehensive web-based database on polyphenol content in foods. It contains more than 37,000 original data points collected from 638 scientific articles published in peer-reviewed journals. The quality of these data has been evaluated before they were aggregated to produce final representative mean content values for 502 polyphenols in 452 foods [19]. The 2D structures, along with their common and IUPAC names,

can be viewed.

2.2.Molecular interaction studies

For studying interactions, we have used iGEMDOCK, a Graphical Environment for Recognizing Pharmacological Interactions and Virtual Screening which combines two methods like structure-based virtual screening and post-screening analysis. It is software for flexible docking of proteins and ligands. First, the program provides interactive interfaces to prepare both the binding site of the target protein and the ligand. Each ligand is then docked into the binding site by using the scoring function. Based on these binding sites and ligands, the software theorize the pharmacological interactions and clusters the screening compounds for the post-screening analysis. Lastly, we can visualize and ranks the screening compounds by merging the pharmacological interactions and energy-based scoring function of iGEMDOCK. It consists of four major modules, docking/screening, post analyzing, molecular visualization, and a parallel processing [20]. Hence, the software was used for protein-ligand docking and Discovery Studio Visualizer 3.5 for visualization of docked complexes.

2.3.Pharmacological and Physicochemical properties

We have used SwissADME server which computes physicochemical descriptors as well as predicts pharmacokinetic properties, druglikeness and medicinal chemistry friendliness of one or multiple small molecules to support drug discovery. It is developed and maintained by the Molecular Modeling Group of the Swiss Institute of Bioinformatics (SIB) [21]. For further phramacokinetic properties, we used admetSAR server which provides the latest and the most comprehensive manually curated data for diverse chemicals associated with known Absorption, Distribution, Metabolism, Excretion and Toxicity profiles. admetSAR is helpful in *in silico* screening, ADMET profiles of drug candidates and environmental chemicals[22].

2.4.Methodology

For a standard docking analysis, library of 76 flavonol compounds were selected and retrieved from Phenol-Explorer server, in SDF file format. These query ligand files were then converted to PDB format, to be used for iGEMDOCK. Also, the SMILES notations for all the ligands were generated using SwissADME server.

2.5.Binding affinity

Structure-based virtual screening and post-screening analysis are emergent tasks in computer-based drug discovery. Combining these two methods to effectively reduce the false positives from a large compound database is considered as a key step in finding the lead compounds. Using iGEMDOCK, the predicted poses can be directly visualized by a molecular visualization tool (DS Visualizer) and refined by post-analysis tools. The Genetic algorithm (GA) parameters were set for standard docking in which population was set as 200, generations as 70 and the number of solutions to be generated as 2 as per the scoring function [23]. The complexes have been analyzed with respect to binding affinity, stability, interacting residues, and forces of attraction between them, etc.

2.6. Prediction of Physicochemical and ADMET properties

The top 10 docked ligands based on energy and pose as ranked by iGEMDOCK were then selected for the prediction of pharmacological properties. The output returned by the server for the query run contains crucial information based upon the following parameters. (i) Physicochemical properties (Molar refractivity, H-bond acceptors, donors) (ii) Lipophilicity (logP values based on different models.) (iii) Water Solubility (iv) Pharmacokinetics (GI absorption, BBB permeability) and (v) Druglikeness. We have also analysed compounds using bioavailability, Radar graph, which enables a first glance at the drug-likeness of a molecule [24]. Secondly, admetSAR server was used for pharmacokinetic based prediction of compounds. The model used for the study was A_BBB_I (Blood-brain barrier) [25]. The server returned results based upon the following parameters: P-glycoprotein substrate/inhibitor, human intestinal absorption, and toxicity to name a few. It also classified compounds as inhibitors of cytochrome P450 enzymes CYP2C9, CYP2C19, CYP2D6 and CYP3A4 and substrates for metabolism by CYP2D6 and CYP3A4 [26].

3. RESULTS AND DISCUSSION

3.1. Molecular interaction studies

A molecular interaction study was performed between all 76 flavonols and MEK1 target to investigate the binding site interaction or pattern. The conformations were generated for all the flavonols and prepared for docking study using iGEMDOCK program known as GA based scoring function. This enables us to compare the binding site cavities along the affinity score or fitness score of all the compounds. Out of 76 compounds we have selected the best 10 flavonols based on the fitness score and desired pharmacokinetic scores. Figure 1(a) showed the crystallographic structure of MEK1 (3EQH) with the drug U0126 ethanolate present at the binding site. Interestingly, b) represent the same location where the flavonol, Isorhamnetin 7-O-rhamnoside compound showed promising specificity at the binding site of the target protein.

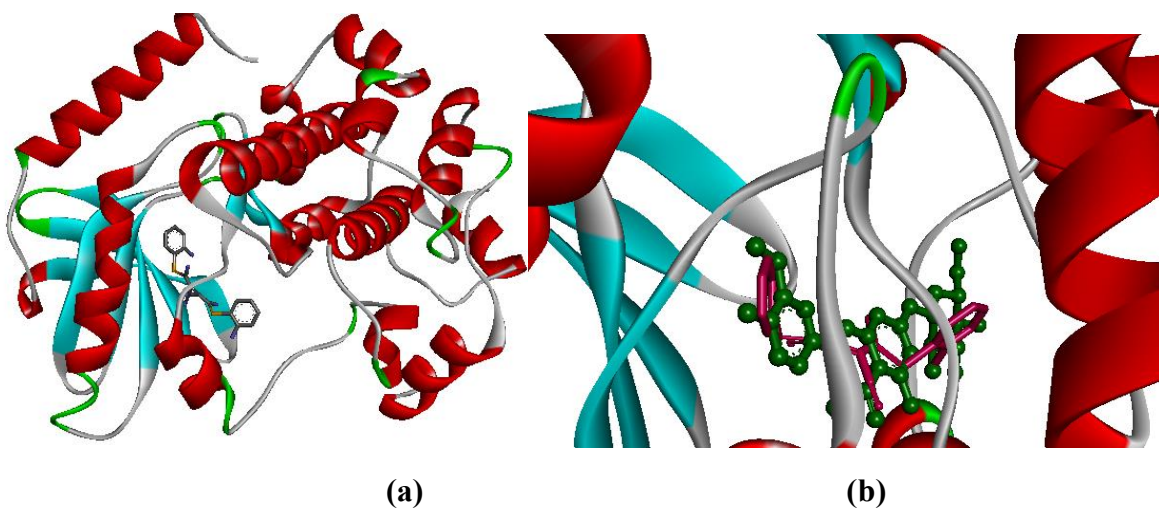


Figure 1: a) Shows crystallographic structure of MEK1 receptor and the drug which is present at the binding site. b) Represents Isorhamnetin 7-O-rhamnoside compound (green, ball & stick)

position at the drug (magenta, stick) binding site. The protein represent as cartoon ribbon while drug is in stick form

We further investigate the solvent accessible surface area (Figure 2) of the binding site with respect to the surrounding amino acids of around 3.5 Å distance. This implies the charged terminal groups of the flavonol has large SAS map area (25.0) where the other groups has a low map surface. This indicates the molecule has a good absorption and distribution property in skin cancer pathway and that gives a good sign for further *in vitro* studies.

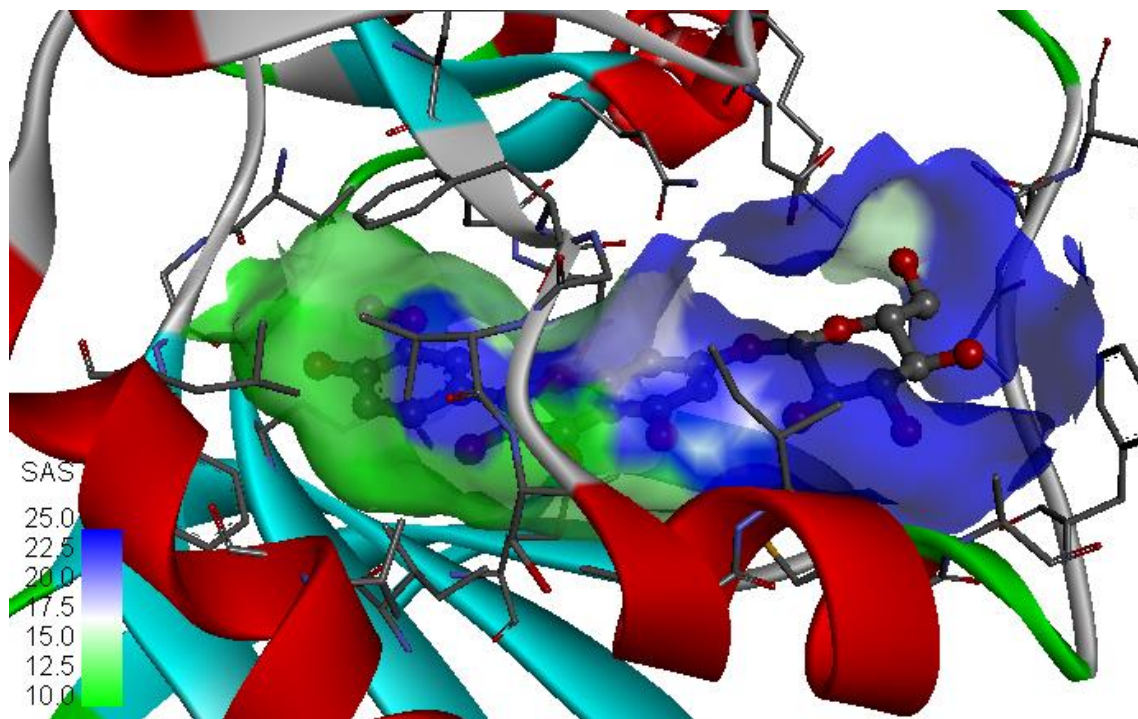


Figure 2: Solvent accessible surface area of the best compound within the binding site of MEK1 receptor

A comparative molecular interaction was explained in Table 1 where the compound names, fitness score, and interacting amino acids and the energy was explained. The interaction score for the top ten compounds have the range between -113.4 and -96.7. We have selected the best compound from the data set as Isorhamnetin 7-O-rhamnoside because of the good binding affinity and promising pharmacokinetic properties. This gives further insight about the need of analysing other toxicity and effective dose parameters of the selected compound for better selectivity and properties. In addition to the reported binding site amino acids (Asp208, 209, Ser212, Val211, Lys97) of MEK1 receptors, we have identified Gly210, Leu215 and Met215 amino acids involved in molecular interaction. Table 3 also indicates the affinities of other flavonol derivatives like Kaempferol, Quercetin and Spinacetin compounds and the respective interactions and specificity.

A detailed view of molecular interaction of the selected flavonol represented in Figure 4, where

mapping of all the binding site amino acids were labelled. The dashed green lines indicate hydrogen bonds (Asp190, Asp 208, Ile216, Gly210, and Ser212). However, Pi-Pi stacked interaction was also shown between Phe209 and the aromatic ring of the compound which depicts the non-covalent interaction involved in ring system. This has played an important role in stabilising the macromolecular structure by the presence of ligand. Apart from Pi-alkyl bonds, an unfavourable bump interaction was also observed by Leu118 which is due to the presence of such Pi-Pi stacking.

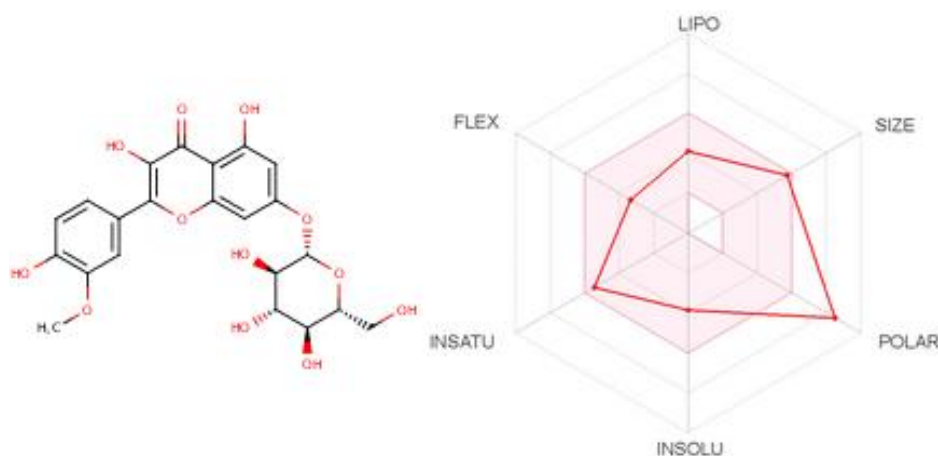


Figure 3: The bioavailability radar plot of Isorhamnetin 7-O-rhamnoside flavonol

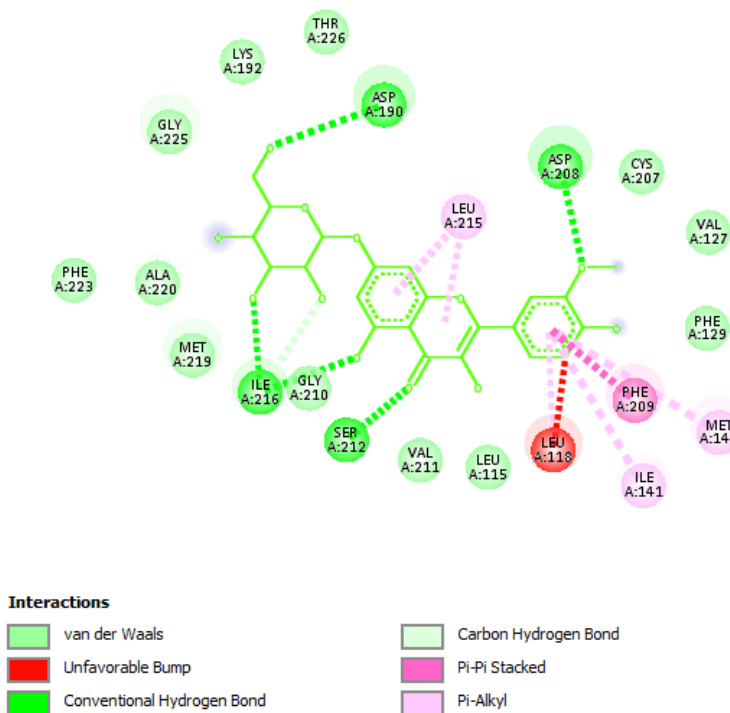


Figure 4: A 2D diagram of relevant molecular interaction of Isorhamnetin 7-O-rhamnoside in the binding site of MEK1 receptor

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3.2. Physicochemical and Pharmacokinetic property identification

The bioavailability of Isorhamnetin 7-O-rhamnoside flavonol was generated using a radar plot shown in Figure 3. This explained lipophilicity, flexibility, insaturation, insolubility, polar and size of the given compound. The thick red lines connected by the nodes indicates the direction towards any of the above properties. Interestingly, except polar group, the remaining properties were present in the form of a well-balanced distribution. This radar plot helps the medicinal chemist to further detect and modify the pharmacophores of the chemical compound for improved bioactivity.

No.	Compound	Energy	Asp-208	Asp-209	Gly-210	Leu-215	Met-219
1.	Isorhamnetin 7-O-rhamnoside	-113.4	- 9.44279	- 6.93587	-6.6333	- 6.24661	- 5.54481
2.	Kaempferol 7-O-glucoside	-106.4	-7.2286	- 4.99159	-5.56181	- 6.25286	- 6.03865
3.	Quercetin 3-O-acetyl-rhamnoside	-102.5	- 7.58349	- 7.93214	-6.84189	- 4.10517	- 6.21847
4.	Isorhamnetin 4'-O-glucoside	-102.3	- 11.6104	- 9.01643	-5.33565	- 5.01165	- 8.21809
5.	Spinacetin 3-O-glucosyl-(1->6)-glucoside	-100.8	- 7.88378	- 10.7513	-6.25861	- 5.11271	- 3.18866
6.	Kaempferol 3-O-(2"-rhamnosyl-galactoside) 7-O-rhamnoside	-98.4	- 11.6941	- 5.93391	-4.79609	- 2.27384	- 12.6853
7.	Kaempferol 3,7-O-diglucoside	-97.4	- 6.41407	- 8.07561	-4.91315	-4.9203	- 5.66587
8.	Quercetin 3-O-glucosyl-rhamnosyl-glucoside	-97.4	- 12.6049	- 7.77273	-4.27855	- 3.56701	3.13727
9.	Isorhamnetin 4'-O-glucuronide	-97.1	- 5.98925	- 3.40363	-3.90314	-9.1812	-13.03
10.	Quercetin 3-O-(6"-acetyl-galactoside) 7-O-rhamnoside	-96.7	- 4.46618	-3.4067	-3.01182	- 0.94903	-11.384

Table 1: Shows molecular interaction table between the 10 best flavonols selected after the screening studies

Physicochemical properties of the selected compounds were calculated and shown in Table 2. The number of rotatable compounds for all the compounds were exceeding 8 which means all the compounds have larger flexible groups and showed a good conformational entropy during interaction studies. Hydrogen bonds acceptors are large enough for all the compounds for

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specificity in binding site. LogP of the compound, Isorhamnetin 7-O-rhamnoside is 1.78 which indicates a moderate hydrophobicity of the compound that may lead to a positive interaction later for activation of the pathway. However, the solvation (LogS) of all the selected compounds shown a range of 2.1-3.5 which indicates similar solvation energy properties in the data set.

Table 3 listed pharmacokinetic properties of the selected flavonols predicted using various servers. This shows a promising property range in terms of BBB, HIA, caco2, Pgp proteins. These properties plays a significant role in determining absorption, specificity, selectivity, toxicity and the role of metabolic activities in different cellular components. All the compounds including Isorhamnetin 7-O-rhamnoside showed a desired property range in various pharmacokinetic training models with an r^2 of 0.85 as correlation coefficient. This gives insight about designing analogues of the compounds for *in vitro* and *in vivo* behaviors. Moreover, Isorhamnetin, Kaempferol, Quercetin and Spinacetin derivatives from food supplements have potent pharmacokinetic properties compared with other flavonoids from different sources.

No	Name	MW	#Rot-bonds	#H-bond acceptors	#H-bond donors	MR	TPSA	Log P	LogS
1.	Isorhamnetin 4'-O-glucoside	478.4	5	12	7	114.63	199.51	1.98	- 2.3177
2.	Isorhamnetin 4'-O-glucuronide	492.39	5	13	7	115.24	216.58	1.52	- 3.3018
3.	Isorhamnetin 7-O-rhamnoside	478.4	5	12	7	114.63	199.51	1.78	- 2.3177
4.	Kaempferol 3,7-O-diglucoside	610.52	7	16	10	140.26	269.43	0.71	- 2.1961
5.	Kaempferol 3-O-(2-rhamnosyl-galactoside) 7-O-rhamnoside	740.66	8	19	11	170.32	308.12	3.46	- 2.6579
6.	Kaempferol 7-O-glucoside	447.37	4	11	6	106.24	193.11	1.57	- 2.5005
7.	Quercetin 3-O-(6-acetyl-galactoside) 7-O-rhamnoside	652.55	8	17	9	150.85	275.5	2.59	- 3.2324
8.	Quercetin 3-O-acetyl-rhamnoside	490.41	5	12	6	118.73	196.35	0.94	- 3.5728

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9.	Quercetin 3-O-glucosyl-rhamnosyl-glucoside	772.6 6	9	21	13	173.7 6	348.5 8	2.38	- 2.2638
10.	Spinacetin 3-O-glucosyl-(1-6)-glucoside	670.5 7	9	18	10	153.5	287.8 9	3	- 2.1589

Table 2: Physicochemical descriptors predicted for the selected compounds

No.	Name	BBB/Probability	HIA/p.	Caco2 permeability, cm/s	P-gp Sub./p.	P-gp I./p. (Chen et al.)
1.	Isorhamnetin 4'-O-glucoside	N/0.9247	Y/0.7769	-0.6481	Y/0.6724	N/0.8575
2.	Isorhamnetin 4'-O-glucuronide	N/0.8812	Y/0.7301	-0.3114	Y/0.6540	N/0.8650
3.	Isorhamnetin 7-O-rhamnoside	N/0.9247	Y/0.7769	-0.6481	Y/0.6724	N/0.8575
4.	Kaempferol 3,7-O-diglucoside	N/0.7248	Y/0.7012	-0.921	Y/0.6341	N/0.8698
5.	Kaempferol 3-O-(2-rhamnosyl-galactoside) 7-O-rhamnoside	N/0.8673	Y/0.8300	-0.646	Y/0.6687	N/0.8625
6.	Kaempferol 7-O-glucoside	N/0.6875	N/0.7589	-0.8431	Y/0.5844	N/0.9365
7.	Quercetin 3-O-(6-acetyl-galactoside) 7-O-rhamnoside	N/0.9147	Y/0.6530	-0.6846	Y/0.7556	N/0.8627
8.	Quercetin 3-O-acetyl-rhamnoside	N/0.7986	Y/0.7896	-0.3819	Y/0.6503	N/0.8458
9.	Quercetin 3-O-glucosyl-rhamnosyl-glucoside	N/0.8589	Y/0.8034	-0.8286	Y/0.7116	N/0.8537
10.	Spinacetin 3-O-glucosyl-(1-6)-glucoside	N/0.9444	N/0.6335	-0.6024	Y/0.7165	N/0.8645

Table 3: Pharmacokinetic property identification of top ten compounds selected from interaction studies

4. CONCLUSION

In the present work, we have studied a number of flavonol compounds from Phenol explorer resource and those are especially from food derivatives. Molecular property calculation was done considering various pharmacokinetic and physicochemical descriptors and identified the desired range and properties of the selected compounds. Moreover, a molecular interaction study was prepared using a powerful GA algorithm and short listed the best 10 flavonols out of 76 compounds. These structures did show a promising binding interaction in comparison with the standard drug of MEK1 protein, which is an important target protein in skin cancer. However, we have proposed Isorhamnetin 7-O-rhamnoside flavonol which is present in berry juice (0.13mg/100 ml) [27] showed a potent binding affinity and molecular interaction for further dose dependant in vitro studies. Thus, the present study sheds light on the need of analyzing large number of flavonoids present in various food content and other sources for a diverse type of cancer based therapeutics.

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6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose

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Study of the Antioxidant Potential of *Staphylococcus succinus* Isolated from *Solanum lycopersicum*

Y. SAIWAL, N. SHIVALE*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

ABSTRACT: *Solanum lycopersicum* (tomato) fruits are known to have antioxidant activity, which helps in curing various diseases, and have been added as an important food item in the diet. A bacterium was isolated from tomato fruit, which possibly may be contributor to its antioxidant potential. The bacterium isolated was identified as *Staphylococcus succinus* by 16s RNA sequencing and confirmed by phylogenetic analysis and MALDI-TOF analysis. Extraction of antioxidants was carried out by ethyl acetate and was studied for its antioxidant activity by various antioxidant assays such as DPPH assay (69.07%), FRAP assay (5.5 µg/ml), Reducing power assay (0.75µg/ml), Lipid peroxidation assay (5.6 µg/ml), Total phenolic content assay (2.1 µg/ml) and Hydrogen peroxide assay (8.62 µg/ml).

KEYWORDS: Tomato, *Staphylococcus succinus*, antioxidants

***Corresponding Author: Ms. Nilima Shivale**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: nilima.shivale@dyatil.edu

1. INTRODUCTION

Human body has an inherent anti-oxidative mechanism and many of the biological functions such as the anti-mutagenic, anti-carcinogenic, and anti-aging responses originate from this property. Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells. Reactive oxygen species are the major source that initiate oxidation and create oxidative stress, resulting in numerous diseases and disorders [1, 2] such as cancer [3], cardiovascular disease [4], neural disorders [5], Alzheimer's disease [6] mild cognitive impairment [7], etc. The damage caused by free radicals or ROS can be prevented and minimized by the antioxidants. Human body uses antioxidants to balance the free radicals.

Sources of Antioxidants are vegetables like green leafy vegetables such as spinach and kale delivering significant amounts of the antioxidant lutein, tomatoes and tomato products carrying the antioxidant lycopene and fruits such as blueberries and other berries, kiwi, prunes, and pink grapefruit.

Tomatoes are known to have antioxidant activity that helps cure various diseases and have been added as important food item in diet. Both raw and processed tomato and tomato products were directly associated with plasma lycopene concentrate thus, it is desirable as dietary choices for vulnerable population groups such as the elderly. A significant number of tomatoes are consumed fresh, some consumers remove the skin and seeds of tomatoes before eating them raw, while some

fresh tomatoes are cooked with or without the skin and seeds. Boiling and baking has been reported to have a relatively small effect on the antioxidant content of tomatoes, whereas, frying significantly ($p < 0.001$) reduced the antioxidant contents [8].

In general, limited data are available on the contribution of the different fractions (skin, pulp and seeds) towards the total amount of the antioxidant components and antioxidant activity of tomatoes. The antioxidant activity of tomato could be contributed by the normal flora bacteria. Therefore, in this study a bacterium was isolated from tomato to find its antioxidant producing capacity.

2. MATERIALS AND METHODS

Fresh, ripe tomatoes from local market field were collected and stored at 4°C overnight to isolate the bacteria. Tomatoes were sliced to remove the outer covering and cut into pieces (approx. 1g) and washed in Buffered peptone water to recover the bacteria. 1-ml of this was added to sterile Brain Heart Infusion (BHI) broth and incubated at 37°C for 48 hours.

2.1. Identification and Characterization of the isolate

Primarily, the isolate was observed under lab microscope to study colony characteristics. Identification of bacteria was done by 16 sRNA sequencing with 1200bp at NCCS-NCMR Labs, Pune. Additionally, MALDI-TOF and Phylogenetic analysis was performed. Complete Bacterial Identification was done by using EzBioCloud Database at NCCS-NCMR LAB, Pune.

2.2. Extraction of Antioxidants

Isolated colonies were obtained on Brain Heart Infusion (BHI) agar plate. The isolate obtained was inoculated in freshly prepared broth and incubated at 37°C for 24 hours. The broth was collected in sterile tubes and centrifuged at 4°C, /10000rpm for 15mins. The supernatant was mixed with equal amount of ethyl acetate and mixed vigorously at 37°C for 1 hour. Ethyl acetate layer was separate and dried in rota-vapour, the final extract was collected in the tubes with extracted sample were stored at 4°C.

2.3. Antioxidant Assays

The efficiency of antioxidants can be measured by *in vitro* methods. *In vitro* methods used were diphenyl- π -picryl-hydrazyl radical scavenging assay (DPPH), Ferric reducing antioxidant power (FRAP), Reducing power assay, Lipid peroxidation assay, Total phenol assay, Hydrogen Peroxide Scavenging Assay. [9, 10].

2.4. DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay

DPPH free radical is reduced to corresponding hydrazine when it reacts with hydrogen donors. 0.3mM stock solution of DPPH was prepared and 2ml of this solution was added to 2ml (0.2ml extract+1.8ml d/w) of extract solution of different concentrations. The mixture was shaken vigorously and incubated in dark for 30 minutes. The decrease in absorbance of the solution was observed using UV-spectrophotometer at 517nm. Ascorbic acid was used as standard (1-10µg/ml).

$$\% \text{ inhibition} = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

2.5.FRAP (Ferric Reducing Antioxidant Power) Assay

FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe^{2+} -TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). 0.2ml of extract + 0.8ml D/W water is added to 3ml FRAP reagent (300mM Acetate buffer + 10mM TPTZ + 10mM Ferric chloride in the ratio of 10:1:1) and the mixture is incubated at 37°C for 30 minutes and increase in absorbance was observed using UV-Spectrophotometer at 593nm. Ascorbic acid was used as standard.

2.6.Reducing Power Assay

Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants). Reducing power can be determined by adding 2.5ml Phosphate buffer (200mM, pH 6.6) and 2.5ml Potassium ferricyanide (30mM) to 1ml (0.2ml extract + 0.8ml d/w water) of extract. The mixture after keeping it in water bath at 50°C for 20 minutes, 2.5ml of Trichloroacetic acid (600 mM) is added to the mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant (approx. 2.5ml) is collected and mixed with 2.5ml d/w water and 0.5ml Ferric chloride (6 mM). The increase in absorbance is observed using UV-spectrophotometer at 700nm. Ascorbic acid was used as standard.

2.7.Lipid Peroxidation Assay

Lipid Peroxidation is oxidative deterioration of lipids containing carbon-carbon double bonds. TBARS assay method is carried out by standard procedures to observe the peroxidation. 1ml (0.1ml extract + 0.9ml d/w water) extract is heated with TBA reagent (20% TCA, 0.5% TBA, 2.5N HCl) in boiling water bath at 95°C for 30 minutes. The mixture is cooled and centrifuged at 1000rpm for 5 minutes. The supernatant was collected and absorbance was measured using UV-spectrophotometer at 532nm.

2.8.Total Phenolic Content

Antioxidative properties of polyphenols arise from their high reactivity as hydrogen or electron donors from the ability of the polyphenol derived radical to stabilize and delocalize the unpaired electron (chain-breaking function) and from their potential to chelate metal ions. 0.5ml of extract is mixed with 0.1ml of Follin-Ciocateu reagent and incubated at room temperature for 15 minutes. Thereafter, 2.5ml of saturated sodium carbonate (48g/100ml) is added and the mixture is incubated at room temperature for 30 minutes. Increase in absorbance is observed using UV-spectrophotometer at 760nm. Gallic acid (1-5µg/ml) was used as standard.

2.9.Hydrogen peroxide (H_2O_2) scavenging Assay

Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4). 1ml extracts in

1ml distilled water were added to a hydrogen peroxide solution (0.4 mL, 40mM). Absorbance of hydrogen peroxide at 230 nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide.

The percentage of hydrogen peroxide scavenging activity can be calculated by formula:

$$\% \text{ Scavenged } [H_2O_2] = \frac{\text{absorbance of control} - \text{absorbance of sample}}{\text{absorbance of control}} \times 100$$

3. RESULTS

Identification and characterization of the isolate: Morphological Characteristics

Sr.No.	Characterisitics	Observed colony characteristics
1	Colour of colony	Yellowish-white
2	Gram Stain	Gram positive cocci
3	Opacity	Opaque
4	Shape	Cocci
5	Size	Pin point
6	Form	Circular
7	Surface	Smooth
8	Elevation	Elevated from centre

Table 1: Morphological Characteristics of isolate obtained from Tomato on sterile BHI agar plate.

The 16 sRNA sequencing identified the organism to be *Staphylococcus succinus* subsp. *succinus* and was confirmed by MALDI-TOF analysis.

Antioxidants Assays

DPPH assay is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the nonradical form DPPH-H.

DPPH free radical scavenging assay shows $69.07 \pm 0.01\%$ inhibition by ethyl acetate extract of the cell free supernatant of *S. succinus*.

The ferric reducing antioxidant power (FRAP) was performed to evaluate the reducing activity of microorganism extract. In this assay, antioxidant acts as reductant by performing reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). FRAP assay of the ethyl acetate extract of *S. succinus* shows presence of $5.5 \pm 0.6 \mu\text{g/ml}$ of antioxidant equivalent to standard ascorbic acid.

Reducing power assay of ethyl acetate extract of the cell free supernatant of *S. succinus* shows $4.79 \pm 0.075 \mu\text{g/ml}$ of antioxidant equivalent to ascorbic acid.

Lipid peroxidation assay carried out at 532nm by ethyl acetate extract of the cell free supernatant of *S. succinus* shows presence of antioxidant in a concentration of $5.6\mu\text{g/ml} \pm 0.05$ equivalence of ascorbic acid.

Total phenolic content assay of ethyl acetate extract of the cell free supernatant of *S. succinus* shows antioxidant activity equivalent to $2.1\mu\text{g/ml} \pm 0.02$ of ascorbic acid.

Hydrogen peroxide assay of ethyl acetate extract of the cell free supernatant of *S. succinus* shows presence of antioxidant equivalent to $8.62\mu\text{g/ml} \pm 0.14$ of ascorbic acid.

Thus all the antioxidant assays suggest that the isolate, *S. succinus* can be used as a potential source of antioxidant.

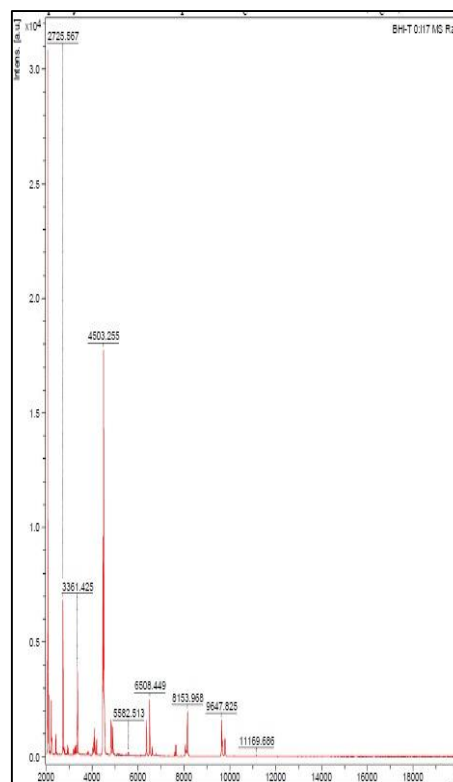
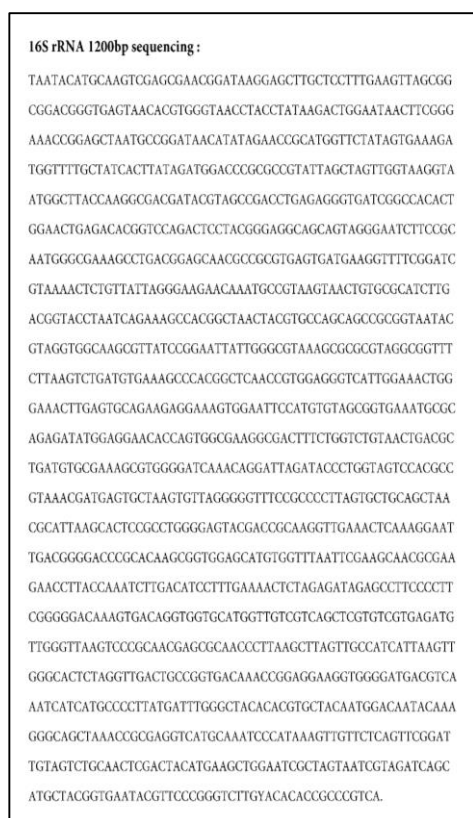


Figure 1: 16 sRNA sequencing of the isolate

Figure 2: MALDI-TOF analysis of the isolate

4. DISCUSSION

Uthairatanakji *et al* (2017) reported the differences in antioxidant potential between organic and conventional tomato fruit. Elbadrawy and Sello, (2016) evaluated the antioxidant activity of tomato peel extracts (petroleum ether, chloroform, ethyl acetate and methanol) along with the reference antioxidant BHT by DPPH assay. Fuentes E, (2017) reported the antioxidant potential in red tomato pulp to be superior to green tomato pulp. Ailton Reis, (2005) have been reported on the involvement of fungi in the wilting of tomatoes. In present study a bacterium was isolated from tomato fruit and its antioxidant potential was determined by various *in vitro* antioxidant assays.

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The isolate was found to be a potent source of antioxidants. The organism was identified to be *S. succinus*, by studying the morphological characteristics, 16sRNA sequencing and MALDI-TOF. G. Mauriello, (2003) have reorted the SOD activity and catalase activity by *S. succinus*. Khusro, (2018) reported the plausible use of *S. succinus* strain AAS2 as biocontrol agent against uropathogens, and due to its antioxidant potential, *S. succinus* strain AAS2 can be further used as pronounced probiotic traits.

5. CONCLUSION

Tomato fruit are major source of antioxidants but in the present study, the antioxidant potential of the isolate viz. *S. succinus* obtained from tomato fruit, was determined by different *in-vitro* assays such as DDPH, FRAP, Reducing Power Assay, Lipid Peroxidation assay, Total phenolic assay and Hydrogen peroxide radical scavenging assay. The isolate exhibited a promising potential of producing antioxidants. The study can be extended further to work on the different varieties/species of tomatoes to evaluate the antioxidant capacity of the organisms associated with it.

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Study of Bioactive Compounds from Methanolic Whole Fruit Extracts of *Annona reticulata* and *Annona squamosa* by GC-MS and Evaluation of Antioxidant Activity

V. AGGARWAL¹, N. JOSHI^{2*}, J. VARGHESE¹

¹B. K. Birla College of Arts, Science and Commerce, Kalyan, Mumbai, India.

²School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

ABSTRACT: The fruits of *Annonaceae* family have enormous medicinal importance. In the present study, methanolic, hydroxymethanolic and aqueous fruit extracts of *Annona reticulata* and *Annona squamosa* were investigated for their therapeutic potential. For this purpose, the total phenolic, flavonoid content and free radical scavenging activity (DPPH, FRAP and ABTS) were studied. Methanolic fruit extract of *A. squamosa* showed highest total phenolic (7.22 ± 0.11 mgGAE/g dry material) and total flavonoid (38.15 ± 0.06 mgQE/gdry material) content. DPPH, ABTS and FRAP result indicated high antioxidant potential of fruit extract. Methanolic fruit extract of *A. reticulata* and *A. squamosa* were subjected to GC-MS analysis to study the important phytochemical compounds responsible for various pharmacological activities. The results revealed the presence of various therapeutically important bioactive compounds like n-Hexadecanoic acid (37.9%), 9,12-Octadecadienoic acid (ZZ)- (36.9%), β -Pimaric acid (7.94%) and 1H-Naphthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a- tetramethyl -methyl ester (8.06%) in *A. reticulata* fruit extract. The major phytochemical compounds present in *A. squamosa* fruit extract were β -Pimaric acid (22.41%), 1-Naphthalenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1, 4 a-dimethyl-6 methylene- (12.03%), Spathulenol (9.15%), 9,12-Octadecadienoic acid (ZZ)- (8.51%) and n-Hexadecanoic acid (6.67%). n-Hexadecanoic acid and 9,12-Octadecadienoic acid (ZZ)- were higher in *A. reticulata* (37.9, 36.9%) than *A. squamosa* (6.67, 8.51%), whereas β -Pimaric acid and 1-Naphthalenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1, 4 a-dimethyl-6 methylene- were higher in *A. squamosa* (22.41, 12.03%) than *A. reticulata* (7.94, 8.06%) fruit extract. These compounds have been reported to show antioxidant, antibacterial, antifungal, antitumor, antidiabetic, hypo-cholesterolemic, anti-atherosclerotic, insecticidal and larvicidal in nature. The present study indicates *A. reticulata* and *A. squamosa* fruits extracts to be a good natural resource for therapeutic active principles and antioxidants.

KEYWORDS: *Annona reticulata*, *Annona squamosa*, antioxidant activity, bioactive compounds, fruit extract, GC-MS

***Corresponding Author: Dr. Neelu Joshi**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

*Email Address: neelu.joshi@dypatil.edu

1. INTRODUCTION

The fruits are considered as a protective food of our diet as they are rich in vitamins, minerals and secondary metabolites of therapeutic importance. The fruits of *Annonaceae* family are rich in phenolic compounds [1]. *Annona reticulata* and *Annona squamosa* are commonly known as “ramphal” and “sitaphal” respectively, in Hindi. These fruits are highly consumed by a large number of populations for their unique delicious taste and nutritional value and are also in great demand in international market of food and dairy industries for making ice-cream, jellies and juices. Apart from being rich in carbohydrates, proteins, vitamins, minerals and amino acids, fruits possess large number of various secondary metabolites like alkaloids, cyclic ketones, flavonoids, glycosides, long chain fatty acids, polysterols, phenols, polyphenols, steroids, tannins and terpenoids [2]. The fruits contain special class of compounds called acetogenins that possess the anticancer properties [3]. Cytotoxic bioactivity of these compounds is due to their potent inhibition of NADH: ubiquinone oxidoreductase (complex 1) of the mitochondrial electron transport system result in inhibition of oxidative phosphorylation and lowering of ATP levels such that cell growth is inhibited [4]. In addition, the fruits have been reported to show antidycentric, vermifuge, antihelminthic, styptic, insecticide and immunosuppressive properties [5]. *Annona* fruits are considered as a new superfruit of 21st century possessing antidiabetic, antimicrobial, antiobese and anticancer properties [6]. The fruit extracts of *A. coriacea*, *A. sylvatica*, *A. diversifolia*, *A. crassiflora* and *A. cherimola* showed high amount of phenols, flavonoids and tannins along with high antioxidant activity [7, 8, 9, 10]. Antioxidants from the natural compounds are always preferred over synthetic antioxidants. The main aim of the study was to explore the antioxidant potential of fruits of *A. reticulata* and *A. squamosa* so that they can be used as an alternative source of natural antioxidants. For this purpose total phenolic, flavonoid content, antioxidant activity and GC-MS based metabolite profiling of fruit extract of two species of *Annonaceae* was carried out.

2. MATERIALS AND METHODS

2.1. Procurement of plant material

The fruits of *A. reticulata* and *A. squamosa* were collected from the local market, Maharashtra region, India. The fruits were taxonomically identified and authenticated by Dr. Jossy Varghese, Dept. of Botany, B.K. Birla College, Kalyan. The fruits were air dried in shade.

2.2. Fruit samples and extraction procedure

The dried fruit were powdered in mixer and grinder. 5 g of fruit powder were extracted using 50 ml solvents; methanol, aqueous: methanol and aqueous two times for 48 hours by the plant tissue maceration method [11]. The extract will be decanted, filtered using Whatman No. 1 paper and concentrated under pressure in a rotary evaporator at 40°C and redissolved in the solvent to determine the concentration. The concentrated extracts were stored in refrigerator at -20°C till further used. Percentage yield *A. reticulata* and *A. squamosa* in different solvent was calculated.

2.3. Chemicals and reagents

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All the reagents used in the study were of analytical grade. Ascorbic acid, aluminium chloride, ammonium persulphate, butylated hydroxytoluene (BHT), folin-Ciocalteu reagent, ferric chloride, gallic acid, glacial acetic acid, potassium persulphate, quercetin, sodium carbonate, sodium nitrite and sodium acetate were purchased from Sigma Chemicals Co., (St. Louis, MO, USA); 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ) and 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Himedia, Mumbai, India.

2.4.Determination of Total Phenolic Content (TPC)

The total phenolic content was determined in fruit extract of both species using the Folin-Ciocalteu reagent as per the standard protocol [12]. 1ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 & 1.0) of the extract or standard (gallic acid) was added to a 25ml volumetric flask containing 9 ml of distilled water. The total phenolic content was determined as mg GAE/g extract on comparison with a standard gallic acid graph. A reagent blank consisting of distilled water was prepared. 1 ml of folin-ciocalteu reagent (1N) was added to the mixture and shaken. After 5 minutes, 10 ml of 7% sodium carbonate was added to the mixture. The volume was then made up to the mark. After incubation for 90 minutes at room temperature, the absorbance against the reagent blank was determined at 765 nm using a UV visible biochrom spectrophotometer. This was compared to the standards curve of Gallic acid concentration and expressed as mg of Gallic acid equivalents per g (mg GAE g⁻¹) of dry powder.

2.5.Determination of Total Flavonoid Content (TFC)

The aluminium chloride colorimetric assay was used to measure the total flavonoid content of the plant extracts [12]. 1ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 & 1.0) of the fruit extract or standard (quercetin) was added to a 10 ml volumetric flask containing 4 ml of distilled water. 0.3 ml of 5% sodium nitrite (NaNO₂) was added to the flask and after five minutes, 0.3 ml of 10% aluminium chloride (Al₂Cl₃) was added. The volume was made up to the mark with distilled water. The solution was mixed and allowed to stand for 30 minutes at room temperature. The absorbance of reaction mixture was measured against the blank at 510 nm using a UV visible spectrophotometer. The total flavonoid content was quantified according to the standard curve prepared for quercetin and concentration of flavonoid was expressed as mg of quercetin equivalents per g (mg QE g⁻¹) of dry powder.

2.6.Antioxidant assays

DPPH radical scavenging activity

Antioxidant activity of the extract was evaluated by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay [13]. 0.1ml of various concentrations (0, 20, 40, 60, 80 & 100µl) of the extract was added to a 2.7ml of methanol and then 0.2 ml of 0.1% freshly prepare methanolic DPPH was added. The mixture was shaken and left to stand at room temperature in the dark. After 30 minutes the decrease in the absorbance was measured at 517nm against a methanol blank. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) of butylated hydroxy toluene (BHT) as a positive control. The concentrations of the samples for 50% inhibition of DPPH (IC₅₀)

were obtained from the graph of DPPH RSA% (radical scavenging activity percentage) versus a concentration of the sample in milligram per milliliter (mg/ml). The ability to scavenge DPPH radical was calculated using the formula:

$$\text{DPPH RSA (\%)} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Where, Abs control is the absorbance of the control solution (sample without extract) and Abs sample is the absorbance of the test sample (sample with extract).

Ferric ion reducing antioxidant power assay

The FRAP method measures the change in absorbance that occurs when the TPTZ (2, 4, 6-tripyridyl-s-triazine)-Fe³⁺ complex is reduced to the TPTZ- Fe²⁺ form in the presence of antioxidant compounds [14]. The FRAP reagent was prepared by mixing 10mM TPTZ solution in 40 mM HCL, 20 mM FeCl₃ and 0.3 M acetate buffer (pH 3.6) in ratio 1:1:10. The above reagents were mixed together and incubated at 37°C for 30 minutes. 1 ml of various concentrations (0, 0.2, 0.4, 0.6, 0.8 & 1.0) of extract were allowed to react with 0.2 ml of the FRAP reagent. The final volume of reaction mixture was made up to 3 ml with distilled water. The reaction mixture was kept in the dark for 30 minutes at 37°C. The absorbance was measured at 593 nm using spectrophotometer. The antioxidant content based on ferric ion reducing ability was calculated using a standard curve of ascorbic acid at 593 nm. The FRAP results was expressed as mg of ascorbic acid equivalent antioxidant capacity per g of dry powder (mg AEAC g⁻¹).

ABTS radical scavenging assay

ABTS assay was carried out using the described method [15]. This method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from one electron oxidation of the synthetic chromophore 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). The stock solution of 7mM ABTS and 2.45mM ammonium persulphate solution was made separately. The working solution was made by mixing the two stock solutions in equal quantity and allowing them to react for 12-16h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an OD of 0.707±0.001 at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Fruit extracts (1ml) were allowed to react with 1 ml of ABTS solution and the O.D. was taken at 734 nm after 6-7 min using the spectrophotometer. All the readings were taken in triplicate. The ABTS scavenging capacity of the extract was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Detection of bioactive compounds by GC-MS

The fruit of both species of *Annona* were subjected to gas chromatography-mass spectral analysis on an Agilent Technologies with model 7890A GC System, equipped with a model JEOL The AccuTOF GCv JMS-T100GCV mass selective detector (EIMS, electron energy, 70eV) and an Agilent chem. Station data system. The GC column was an HP-5ms fused silica capillary with a EB-5 (5% phenyl methyl polysiloxane), film thickness of 0.25µm, a length of 30 m and an internal diameter of 0.25mm. The carrier gas was helium with a flow rate of 1.0 ml/min. Inlet temperature was 250°C and MSD detector temperature was 270°C. The mass spectrometer was

operated in electron impact ionization (EI) mode with 70eV energy. The mass range was 50-700 a.m.u. and the ion source temperature was 200°C. The GC oven temperature program was used as follows. 80°C initial temperature for 2 min then programmed at 10°C/min to 270°C and held for 10 min. Concentrated methanolic fruit sample of both species were used. 1µl injection using a splitless injection technique was used. Identification of components was achieved based on their retention indices (RI) and by comparison of their mass spectral fragmentation patterns with those reported in the literature [16] and stored on the MS libraries [NIST 05; Mass Finder database (G1036A, revision D.01.00 and Wiley 7 Mass Finder)].

2.7. Statistical analysis

The Statistical analysis was performed using SPSS 14.0 software. All data were expressed as the mean $\pm$ S.D. (Standard Deviation) and all the experiments were performed in triplicate. An analysis of variance (ANOVA) was performed to assess the statistical significance of the differences between the samples. Duncan's multiple range test (DMRT) was performed for comparison of means, considering a confidence level of 95% ($p < 0.05$). Linear regression analysis R^2 was performed on the standard and test extracts. The IC_{50} values were calculated by linear regression analysis using Microsoft Excel. Linear correlation was performed to assess the relationship between the total phenols, flavonoids and antioxidant assays following Pearson's method.

3. RESULTS AND DISCUSSION

3.1. Percentage of extraction yield

The percentage yield was highest in hydroxymethanolic extract in *A. reticulata* (14.6%) and methanolic extract in *A. squamosa* (11.1%) (Table1). Thus, hydroxymethanol served as the best solvent for extracting principle components of *A. reticulata* and methanol considered as best solvent for extraction of *A. squamosa* in the present study.

Sample	Colour of extract	Yield in different solvent systems (%)	
		M	AQ:M (1:1)
<i>A. reticulata</i> fruit	Light green	AQ	
		13.26	14.60
<i>A. squamosa</i> fruit	Dark brown	8.72	
		11.10	10.92
		8.02	

Table 1: Percentage extraction yield of *A. reticulata* and *A. squamosa* fruit in different solvent systems

Methanol (M), Aqueous: Methanol (AQ: M) and Aqueous (AQ).

3.2. Total Phenol and Flavonoid content (TPC and TFC)

The fruits belonging to genus *Annona* are rich in polyphenolic compounds [17]. The phenols and flavonoids have the ability to reduce free radical damage and have potential health benefits in diseases caused by oxidative stress by absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [18]. The present investigation indicated the good

amount of phenol and flavonoids present in fruit extract. Values of TPC and TFC were significantly different ($p < 0.05$) across the two species and the solvent system used. As compared to other solvents methanolic extracts showed maximum values of TPC (7.22 ± 0.11 mg GAE/g) and TFC (38.15 ± 0.06 mg QE/g) in *A. squamosa* and TPC (1.25 mg GAE/g) and TFC (12.63 ± 0.49 mg QE/g) in *A. reticulata* (Table 2). A high TPC of 22.17 ± 0.02 mg GAE/g of dry weight and TFC of 5.13 ± 0.02 mg QE/g were found in methanolic fruit extract of *A. squamosa* [19], which was in contrast to our study and this difference might be due to seasonal and geographical variations. Similar to our results, several other researchers have also reported high TPC and TFC in the different fruit extracts. A TPC of 2.5, 1.7, 1.2 and 16.2 gGAE/100g of dry weight were found in whole fruit of *Ficus carica*, *Gardenia jasminoides*, *Psidium guajava* and *Gordonia axillaris* [20] and high TFC of 107, 142, 152 mg catechin/ 100g of dry weight were found in fruit extract of different species of *A. diversifolia* [8].

Analysis	<i>A. reticulata</i> fruit (ARF)			<i>A. squamosa</i> fruit (ASF)		
	M	AQ:M	AQ	M	AQ:M	AQ
TPC	1.25 ± 0.01^c	1.00 ± 0.02^c	0.48 ± 0.05^c	7.22 ± 0.11^a	5.15 ± 0.06^b	1.05 ± 0.04^d
TFC	12.63 ± 0.49^d	2.57 ± 0.14^c	0.73 ± 0.01^f	38.15 ± 0.06^a	25.07 ± 0.06^b	13.3 ± 0.09^c

Table 2: Total phenolic Content (TPC) and total flavonoid Content (TFC) in fruit extract of *A. reticulata* and *A. squamosa*

Values are average of three replicates $\pm$ standard deviation. Means followed by different letters in a row are significantly different ($p < 0.05$), DMRT.

TPC value expressed as mg GAE/g of dried material.

TFC value expressed as mg QE/g of dried material.

3.3. Antioxidant studies

DPPH assay

DPPH assay has been the most widely used assay to evaluate antioxidant activity of plants. The results of the present study showed that the scavenging effect of whole fruit extract (sample) and BHT (standard) on the DPPH radical increased sharply with the increasing concentration of sample in both the species of *Annona*. In both species highest percentage of free radical scavenging activity (RSA) was found in methanolic extract followed by hydroxymethanolic and aqueous. It was $94.46 \pm 0.17\%$ and $69.50 \pm 1.21\%$ in ASF and ARF, respectively which was much higher than that recorded for synthetic antioxidant (BHT), a positive control ($77.57 \pm 0.38\%$). Our findings were in agreement with previous study that also found high free radical scavenging activity in fruit extract of *A. squamosa* [19]. From a plot of concentration against percentage of inhibition, a linear regression analysis was performed to determine the IC_{50} value (the extract concentration that could scavenge 50% of the DPPH radicals). Lowest IC_{50} value indicated better radical scavenging activity. In the present study, methanolic fruit extract of *A. squamosa* showed great percentage of DPPH free radical scavenging activity (Table 3).

ABTS assay

ABTS is another important assay used to measure antioxidant activity. Ability of *A. reticulata* and *A. squamosa* fruit extracts to scavenge the blue green colored ABTS radical cation was measured relative to the radical scavenging ability of gallic acid. Around 95% inhibition of ABTS free radicals was shown by methanolic extract of both the species of *Annona* (Table 3) indicating rapid scavenging activity. Similar to our results ABTS assay based high antioxidant activity in fruit pulp with TEAC values of 4.4 in *A. cherimola* ethanolic extract [10] and 12.5 in fruit pulp of *A. squamosa* methanolic extract has been reported earlier [20]. High TEAC values were also found in whole fruit extracts of *Ficus carica*, *Gardenia jasminoides*, *Psidium guajava* and *Gordonia axillaris* [20].

Assay	<i>A. reticulata</i> fruit (ARF)			<i>A. squamosa</i> fruit (ASF)		
	M	AQ:M	AQ	M	AQ:M	AQ
DPPH	69.5±1.21 ^c	61.16±1.58 ^c	46.31±0.35 ^f	94.46±0.17 ^a	89.06±0.20 ^b	65.49±0.20 ^d
RSA (%)						
IC ₅₀ (mg/ml)	0.735±0	0.871±0.01	1.119±0.01	0.872±0	0.991±0	1.623±0
ABTS	95.46±0.43 ^a	84.36±0.59 ^c	52.19±0.51 ^d	95.25±0.47 ^a	85.64±0.29 ^b	34.08±0.62 ^e
I (%)						
FRAP	14.04±0.25 ^a	11.04±0.08 ^c	1.15±0.13 ^f	13.77±0.11 ^b	8.99±0.11 ^d	5.19±0.05 ^e
(mgAEAC/g)						
Control						
BHT	77.57±0.38					
IC ₅₀ (mg/ml)	0.705					
Gallic acid I (%)	94.68±0.19					

Table 3: Antioxidant activity of *A. reticulata* and *A. squamosa* fruit extract

Values are average of three replicates ± standard deviation. Means followed by different letter in a row are significantly different (p<0.05), DMRT.

positive control

ABTS antioxidant activity expressed in inhibition percentage (%) and gallic acid used as control.

FRAP result expressed as mg Ascorbic acid Equivalent Antioxidant Capacity/g of dried material.

FRAP assay

FRAP assay is based on the ability to reduce ferric (III) to ferrous (II) ions. FRAP assay treats the antioxidants present in the sample as reductant in a redox linked colorimetric reaction [21]. Out of all the solvent tested, methanolic peel extract showed highest FRAP values in both the species (Table 3) with *A. reticulata* (14.04±0.25 mg AEAC/g) and for *A. squamosa* (13.77±0.11 mg AEAC/g). High free radical scavenging activity as revealed by three assays could be attributed to the presence of antioxidant compounds in fruit extract. In the present study, methanolic extract

showed highest free radical scavenging activity based on all the three assays. In previous studies different free radical scavenging activity assay showed varying results in the same set of extraction solvents [19, 20].

3.4. Correlation between TPC, TFC and antioxidant activity

To establish the relationship between TPC, TFC and antioxidant assays (DPPH, ABTS and FRAP) correlation analysis was performed (Table 4). TPC and TFC showed positive correlation with not all but one or the other antioxidant activity assay. TPC showed positive correlation with FRAP in *A. reticulata* fruit extract whereas TPC showed positive correlation with TFC, DPPH and ABTS in *A. squamosa* fruit extract.

Results of all three antioxidant assays revealed that the fruit extract possess free radical scavenging activity and there was a significant correlation ($p < 0.05$) between DPPH and ABTS assay in *A. squamosa* fruit extract and in *A. reticulata* significant correlation ($p < 0.05$) was found between TPC and FRAP assay. In some studies the correlation between TPC and antioxidant activity was absent, however majority of the studies showed a positive correlation [22, 23].

	TPC	TFC	DPPH	ABTS	FRAP
ARF					
TPC	1	-.575	-.093	-.088	.829(**)
TFC	-.575	1	.310	-.001	-.477
DPPH	-.093	.310	1	.407	-.163
ABTS	-.088	-.001	.407	1	-.303
FRAP	.829(**)	-.477	-.163	-.303	1
ASF					
TPC	1	.031	.260	.137	-.174
TFC	.031	1	-.469	-.294	.210
DPPH	.260	-.469	1	.875(**)	-.417
ABTS	.137	-.294	.875(**)	1	-.525
FRAP	-.174	.210	-.417	-.525	1

Table 4: Correlation (r) between total phenolic content (TPC), total flavonoid content (TFC) and different antioxidant capacity parameters (DPPH, ABTS and FRAP) of methanolic fruit extract of *A. reticulata* (ARF) and *A. squamosa* (ASF)

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

3.5. GC-MS metabolite profiling

In order to identify the active principles of *Annona* fruit, GC-MS profiling was carried out. The compounds were identified on the basis of retention times (RT) and mass-to-charge ratios (m/z).

The result revealed the presence of various phytochemicals including alkaloids, essential oils, fatty acids, flavonoids, phenols, terpenoids and tannins.

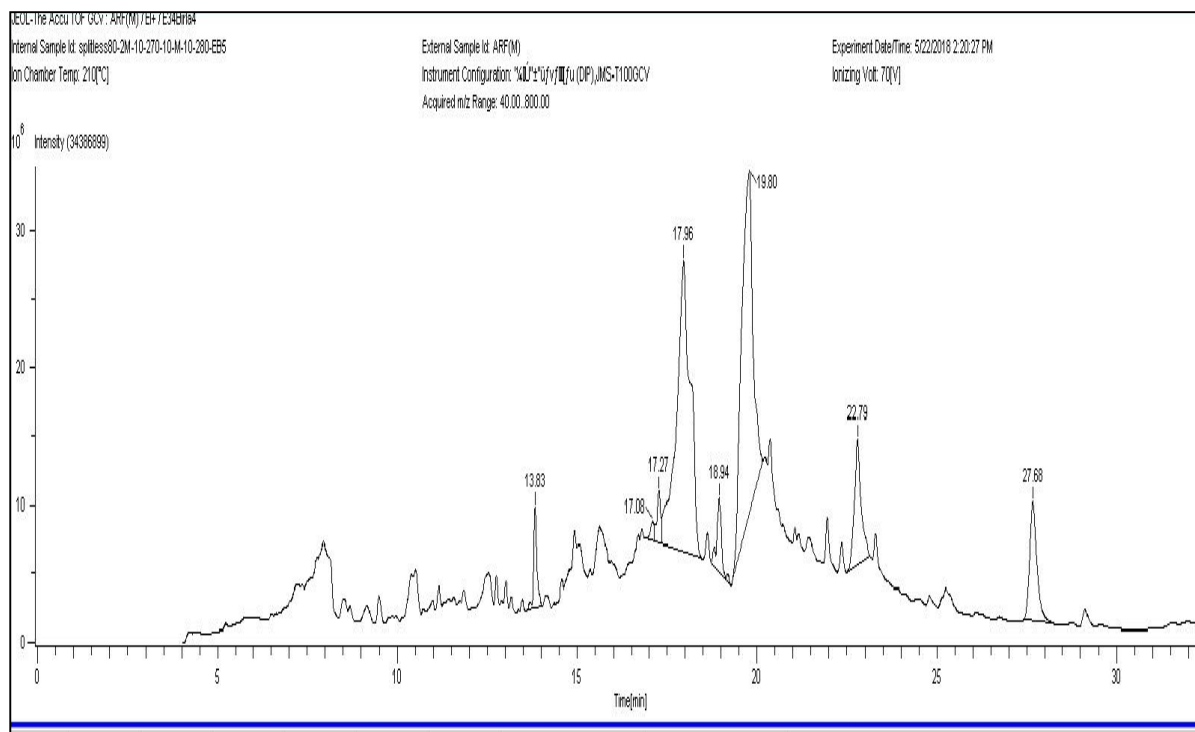


Figure 1: GC-MS chromatogram of methanolic extract of *A. reticulata* fruit

The chromatogram obtained for methanolic fruit extract showed a total of eight compounds in *A. reticulata* and twenty-one compounds in *A. squamosa* (Figure 1, 2). The chromatogram revealed that the compounds with major retention peaks (13.83, 17.08, 17.27, 17.96, 18.94, 19.80, 22.79 and 27.68) in *A. reticulata* fruit were Spathulenol, d-Mannose, Ribitol, n-Hexadecanoic acid, Cyclopropanebutanoic acid, 9,12-Octadecadienoic acid (Z,Z)-, β -Pimaric acid and 1H-Naphthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a-tetramethyl -methyl ester (Figure 1, Table 5). The peaks found at retention time 17.96 and 19.80 were abundant peaks among all and the major compounds identified against them were n-Hexadecanoic acid (37.90%) and 9, 12-Octadecadienoic acid (Z,Z)- (36.90%). These compounds have been reported to possess antioxidant, antidiabetic, hypo-cholesterolemic, nematocide, pesticide, anti-androgenic, 5- α reductase inhibitor, antiarthritic, hepatoprotective, anti-histaminic, anti-coronary, anti-eczemic, anti-acne acidifier, antitumor, apoptotic, cytotoxic, anti-inflammatory, insectifuge and anti-neosporal in properties [24,25, 26].

The chromatogram of *A. squamosa* fruit extract showed major peaks at retention time (9.00, 13.82, 17.78, 19.46, 21.92, 22.58 and 25.33) and their mass-to-charge ratio (m/z) revealed the presence of 1,2-Benzenediol, Spathulenol, n-Hexadecanoic acid, 9,12-Octadecadienoic acid (Z,Z)-, 1-Naphthenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentanyl)-1,4a-dimethyl-6-methylene-, β -Pimaric acid and Androstane-3,11-diol,(3 β -5 α -11 β)- (Figure 2, Table 6).

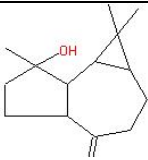
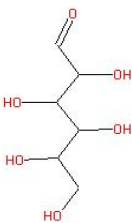
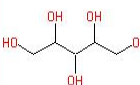
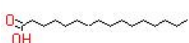
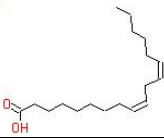
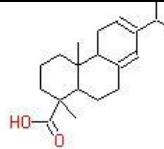
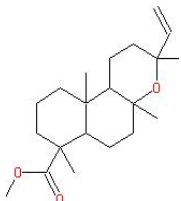
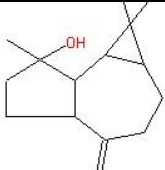
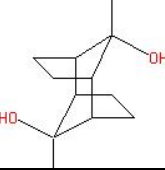
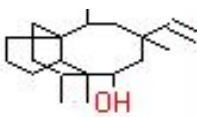
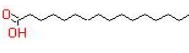
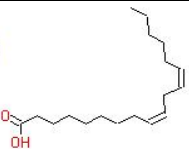
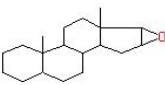
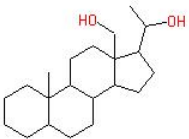
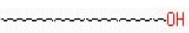
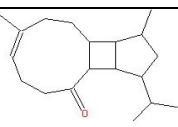
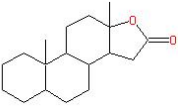
No.	Name of Compound	M.F.	M.Wt. (g/mol)	R.T. (min)	Peak Area (%)	Expected Structure	Biological activity*
1.	(-)-spathulenol, 1H- cyclopropa[e]azulen- 7-ol, decahydro-1,1,7- trimethyl-4- methylene-	C ₁₅ H ₂₄ O	220.35	13.83	3.25		Antioxidant, Antiproliferative, Immunomodulatory
2.	d-Mannose	C ₆ H ₁₂ O ₆	180.15	17.08	0.697		Nutritional supplement of sugar
3.	Ribitol	C ₅ H ₁₂ O ₅	152.14	17.27	1.854		Part of chemical structure of riboflavin and flavin mononucleotide
4.	n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	256.42	17.96	37.90		Antioxidant, Antidiabetic, Hypo- cholesterolemic, Nematicide, Pesticide, Anti- Androgenic
5.	Cyclopropanebutanoic acid	C ₂₅ H ₄₂ O ₂	374	18.94	3.37		Antioxidant
6.	9,12-Octadecadienoic acid (Z,Z)-	C ₁₈ H ₃₂ O ₂	280.44	19.80	36.90		Acidifier, Cardioprotective Antitumor, Anti- inflammatory, Anti-neosporal
7.	β-Pimaric acid	C ₂₀ H ₃₀ O ₂	302.45	22.79	7.94		Antibacterial, Anti-atherosclerotic
8.	1H-Napthol[2,1- b]pyran-7-carboxylic acid, 3- ethenyldodecahydro- 3,4a,7, 10a- tetramethyl -methyl ester	C ₂₁ H ₃₄ O ₃	334	27.68	8.06		Prevent abscess, Abortifacient

Table 5: List of bioactive compounds detected in methanolic fruit extract of *A. reticulata* by GC-MS analysis



β -Pimaric acid was found to be maximum in the extract and reported to show antibacterial and anti-atherosclerotic activity [32, 33]. Other compounds present in *A. squamosa* fruit extract were listed in Table 6 with reference to their biological activities.

Oc1ccccc1O

3.	(-)-Spathulenol, 1H-cyclopropa[e]azulen- 7-ol, decahydro-1,1,7- trimethyl-4-methylene-	$C_{15}H_{24}$ O	220. 35	13.8 2	9.1 5		Antioxidant, Antiproliferative, Immuno- Modulatory
4.	9,10-Dimethyltricyclo [4,2,1,1(2,5)]decane- 9,10- diol	$C_{12}H_{20}$ O ₂	196. 28	15.6 0	2.9 9		Antifungal, Antioxidant
5.	2,4,7,14-Tetramethyl-4- vinyl- tricyclo[5.4.3.0(1,8)]- tetradecan-6-ol	$C_{20}H_{34}$ O	290. 48	16.6 8	1.3 9		Biodiesel
6.	n-Hexadecanoic acid	$C_{16}H_{32}$ O ₂	256. 42	17.7 8	6.6 7		Antioxidant, Antidiabetic,Hypo- cholesterolemic, Nematicide, Pesticide, Anti- Androgenic
7.	9,12-Octadecadienoic acid(ZZ)-	$C_{18}H_{32}$ O ₂	280. 44	19.4 6	8.5 1		Acidifier, Antitumor
8.	5α,14β- Androstane,16α,17α- epoxy-	$C_{19}H_{30}$ O	274. 44	20.3 3	2.2 8		Antioxidant, Antitumor
9.	Pregnane-18,20-diol,(5α)-	$C_{21}H_{36}$ O ₂	320. 50	20.5 5	0.2 9		Antieczematic, Anti-inflammatory
10.	1-Heptatriacotanol	$C_{37}H_{76}$ O	536. 99	20.7 7	0.2 0		Anti-Hyper cholesterolemic
11.	4,14-Dimethyl-11- isopropyltricyclo[7.5.0.0(10,14)]tetradec-4-en-8- one	$C_{19}H_{30}$ O	274	20.9 2	1.4 7		Biofuel or Bio-oil
12.	17-Oxaandrostane-16-one, (5α)-	$C_{18}H_{28}$ O ₂	276. 41	21.4 8	2.3 0		Antioxidant, Antitumor

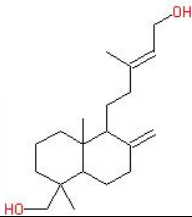
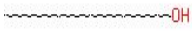
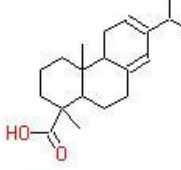
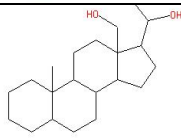
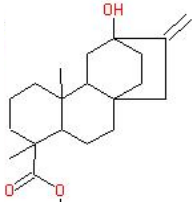
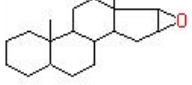
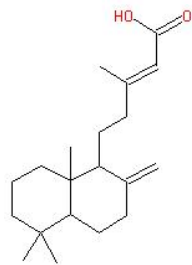
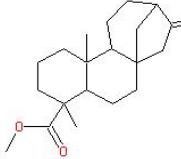
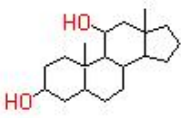
13.	1- Naphthenemethanol, decahydro-5-(5-hydroxy-3-methyl-3-pentanyl)-1,4a-dimethyl-6-methylene-	$C_{20}H_{34}O_2$	306.48	21.92	12.03		Insecticidal, Larvicidal
14.	1-Heptatriacotanol	$C_{37}H_{76}O$	536.99	22.28	1.89		Anti-hypercholesterolemic
15.	β -Pimaric acid	$C_{20}H_{30}O_2$	302.45	22.58	22.41		Antibacterial, Anti-atherosclerotic
16.	Pregnane-18,20-diol,(5 α)-	$C_{21}H_{36}O_2$	320.50	22.92	3.72		Antieczematic, Anti-inflammatory
17.	Isosteviol methyl ester	$C_{21}H_{32}O_3$	332.47	23.37	2.16		Antioxidant, Antidiabetic
18.	5 α ,14 β -Androstane,16 α ,17 α -epoxy	$C_{19}H_{30}O$	274.44	23.76	4.49		Antioxidant, Antitumor
19.	2-Pentenoic acid, 5-(decahydro-5-5-8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-	$C_{20}H_{32}O_2$	304.46	24.31	2.53		Analgesic, Antitumor
20.	Kaur-16-en-18-oic acid, methyl ester, (4 β)-	$C_{21}H_{32}O_2$	316	24.70	3.90		Antioxidant, Cytotoxic
21.	Androstane-3,11-diol,(3 β -5 α -11 β)-	$C_{19}H_{32}O_2$	292.45	25.33	5.43		Antioxidant

Table 6: List of bioactive compounds detected in methanolic fruit extract of *A. squamosa* by GC-MS analysis

M.F. – Molecular formula, M.Wt.- Molecular weight, R.T.- Retention time

*Biological Activity source: Dr. Duke's Phytochemical and Ethnobotanical Database (1992) [34], Science Direct and WorldCat.org, Springer link [online database].

4. CONCLUSION

Methanol proved to be the best solvent in terms of extraction yield, total phenol, flavonoid content and antioxidant activity in fruit extract of both the species of *Annona*. *A. squamosa* fruit extract were richer in flavonoids and showed greater free radical scavenging ability as compared to *A. reticulata* fruit extract. GC-MS based metabolite profiling revealed the presence of therapeutically important bioactive compounds like n-Hexadecanoic acid, 9,12-Octadecadienoic acid (ZZ)-, β -Pimaric acid, 1H- Naphthol[2,1-b]pyran-7-carboxylic acid, 3-ethenyldodecahydro-3,4a,7, 10a-tetramethyl -methyl ester, 1-Napthalenemethanol,decahydro-5-(5-hydroxy-3-methyl-3-pentenyl)-1,4a-dimethyl- 6 -methylene- and Spathulenol. These compounds have been reported to show high antioxidant, antibacterial, antifungal, antitumor, antidiabetic, hypo-cholesterolemic, anti-atherosclerotic, insecticidal and larvicidal activity. Hence, it can be concluded that *Annona* fruits can be a potential natural resource for pharmaceutically important bioactive compounds. It is recommended to undertake further studies to find out bioactivity and toxicity profile of these compounds.

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6. CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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Preliminary Investigation on Repurposing of NSAIDs by Assessing Cytotoxic Effect on Hepatic Cell Line

A. SHAIKH, H. GANGRADE, N. AGRAWAL, M. BHORI, K. SINGH*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Sector -15,
Plot No-50, CBD Belapur, Navi Mumbai-400614
Mumbai, Maharashtra, India.

ABSTRACT: Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are the most commonly used drugs for curing pain, fever, and inflammation. These drugs can be availed by consumers without any medical practitioner's prescription because they are innocuous in nature. Research dealing with the use of drug combinations via repurposing is growing rapidly due to the opportunities they create to increase the therapeutic effect and to reduce the frequency or magnitude of undesirable side effects. Thus, in present research investigation, we attempted to evaluate the cytotoxic effect of two different NSAIDs, one of them being aspirin, which is prescribed for the lifetime of patients suffering from cardiovascular disorders or carrying heart implant and diclofenac which is another most commonly used painkiller. Using *insilico* tools, we determined interactions of two NSAIDs with its active site on the Cyclooxygenase (COX-2) enzyme using AutoDock as docking tool. AutoDock displayed the binding energy for diclofenac and aspirin to be -7.2kcal/mol and -5.6kcal/mol respectively. Cytotoxicity of aspirin and diclofenac was investigated on the normal hepatic cell line (WRL 68) using MTT and Trypan blue assay. Effect of aspirin and diclofenac were assessed at different concentrations ranging from 0.1-10mM and 1-1000μM respectively. IC₅₀ was found to be 220μM and 1.32mM for diclofenac and aspirin respectively. Further, IC₅₀ dose was used to investigate the cytotoxic effect of these drugs when used in combination i.e., 0.25, 0.5, 2 and 4 times the IC₅₀ concentration. Combinational analysis of cytotoxicity induced by both the drugs showed synergism, which was verified using CompuSync platform with CI values ranging from 0.48 to 0.76. In order to ascertain the cytotoxic effect of aspirin and diclofenac alone or in combination on hepatic cancers, it is of prime importance to validate its effects on normal cells. This would help in choosing not only the proper drug but also the safe combination in which they can be used to amplify their repurposed therapeutic effect.

KEYWORDS: NSAIDs, Aspirin, Diclofenac, Cytotoxicity, *insilico* studies

***Corresponding Author: Dr. Kanchanlata Singh**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Sector -15, Plot No-50, CBD Belapur, Navi Mumbai-400614

*Email Address: skanchan19@gmail.com; Contact: +91-9594899029.

1. INTRODUCTION

Non-Steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that is used as analgesic, antipyretic, anti-thrombotic and, in higher doses, decreases inflammation. NSAIDs are known to inhibit the activity of an enzyme called as Cyclooxygenase (COX-1 and/or COX-2). COX, is an

enzyme that is responsible for the synthesis of prostaglandins (PG) which in turn is involved in inflammation. COX enzyme is also responsible for production of thromboxane from arachidonic acid which is involved in blood clotting mechanisms. COX has two isoforms, COX-1 and COX-2. Aspirin (2-Acetoxybenzoic acid) or acetylsalicylic acid (ASA), is used in the treatment of pain, inflammation, and fever. It is also used as a blood thinner in various therapies to prevent myocardial infarction, stroke and transient ischemic episodes. Diclofenac (2-(2, 6-dichloranilino) phenylacetic acid, DIC) is a NSAID's that is taken to reduce inflammation and serve as an analgesic. Studies on colorectal cancer have reported NSAIDs and selective COX-2 inhibitor possessing chemo preventive action against cancer. Most NSAIDs prolonged use has a notable low mortality rate in colorectal cancer [1, 2].

Combination therapy or polytherapy uses more than one medication or modality to treat a single disease. Generally, these strategies include pharmaceutical or medical therapy whereas drug repositioning (also known as drug repurposing, re-profiling, re-tasking or therapeutic switching) is the application of known drugs and compounds to treat a different disease. Present research investigation is preliminary attempt to establish drug repurposing of ASA and DIC, by studying their cytotoxicity on normal hepatic cell line.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

WRL 68 was procured from National Centre for Cell Science (NCCS), Pune. ASA, DIC salt powder and Dulbecco's Modified Eagle's Medium (DMEM) were acquired from Sigma. Fetal bovine serum (FBS) and Trypsin - EDTA (1X 0.25%) was obtained from GIBCO. DMSO (Dimethyl sulfoxide) and sodium pyruvate (100mM) was obtained from HiMedia. Penicillin-Streptomycin (10,000Units/ml-10,000µg/ml) was obtained from cell clone.

2.2. Docking with autodock

Docking of ASA and DIC was done on COX-2. PDB structure for COX-2 was retrieved from RCSBPDB. Structure for ASA and DIC was obtained from PubChem compounds which was optimized using Frog2 online program [3]. SPDBV was used to optimize receptor [4]. The protein i.e. receptor was opened in MGL tools [5] which served as Graphical User Interface (GUI) for AutoDock. Non-polar hydrogens were merged and atoms were assigned AD4 types. Kollman charges were added as template values for each amino acid that derived from the corresponding electrostatic potential using quantum mechanics. This file was saved as "Receptor.PDBQT". Similarly, on the ligand in MGL tools, the gasteiger charges were computed on the basis of electronegative equilibration. Further, the root was detected in root tree and a number was set with a value of less than 10. File was saved as "Ligand.PDBQT". Using ligand and receptor PDBQT files the grid was generated and offset was noted down along X, Y and Z directions. After which these values were saved in config file containing grid dimensions. Docking was done using command "vina.exe -config config.txt -log log.txt". Results were generated in a single file and visualized using USCF Chimera and hydrogen bond was calculated [6]. The COX-2 receptor was opened in UCSF Chimera docked poses, and was visualized using ViewDock tool. Hydrogen

Bonds were calculated which formed between ligand and residue. Finally distance showing ligand interaction with the receptor was calculated for analyzing the docked poses.

2.3.Cell line studies

2.3.1.Cytotoxicity assay using MTT

WRL-68 cell line was maintained in DMEM media supplemented with 10% FBS, 100 U/ml of penicillin- 100µg/ml streptomycin solution and 1 mM sodium pyruvate in 5% CO₂ at 37°C. Once healthy monolayer cells were obtained, existing media was discarded and cells were then washed with 1X PBS (pH – 7.4). 1X trypsin was used during subculture and harvested cells were collected in fresh complete medium. Cytotoxic effect of the drugs was determined using MTT assay as per modified Mosmann protocol [7]. 4500 cells per 200µl of complete medium were seeded in each well of a 96-well plate in triplicate and the cells were incubated at 37°C in CO₂ incubator for 24 hours. Further, stock drug i.e. DIC and ASA was added in appropriate volumes at various concentrations and incubated for next 24 hours. Control, vehicle control and negative control were also maintained throughout the experiment. The final volume of stock concentration did not exceed 1% (i.e. 2µl). On the next day, 0.5mg/ml in 20 µl from MTT stock solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) was added in each well and was allowed to get reduced into insoluble formazan crystals by keeping it at 37 °C for 4 hours. Further whole content of well was discarded carefully without losing the purple formazan crystals and then was dissolved by adding 100µl of DMSO. The absorbance was read using Bio-Rad microplate reader (version 680) at 570nm. The experiment was repeated three independent times in triplicates and percent viability was calculated.

2.3.2.Morphological studies

Approximately 1×10^5 cells/ml of complete medium were seeded in each well of 6-well plate. After 24 hours, cells were treated with DIC and ASA at various concentrations. Separate wells of control and vehicle control were also maintained throughout the experiment. Inverted microscopy was performed using Nikon eclipse TS100 to understand the effect of both the drugs on the morphology of the cells.

2.3.3.Cell viability assay using dye exclusion method

Trypan blue viability assay was performed as per the protocol described by Strober [8]. 1×10^5 cells/ml were plated into each well of a six well plate and incubated at 37°C for 24 hours. Cells were then treated with various concentrations of DIC and ASA for 24 hours with appropriate controls being maintained. Fresh culture medium was exchanged before addition of the drugs. After incubation, all cells were harvested, mixed with trypan blue (1:10) and counted using neubauer's improved hemocytometer. Total cell density and percentage viable cells were then calculated.

2.4.Drug combination setup

The combination studies were performed in 96-well plate, in which 36 samples were taken in

account out of which one served as control, and other 10 wells were treated with 5 different concentration of each drug alone. Rests of the wells were treated with 25 different combination of the drug. Of the five concentration tested for each drug, the middle dose was approximately equal to the IC_{50} ; two higher doses (two and four times the IC_{50}) and two lower doses (0.25 and 0.5 times the IC_{50}) were also tested. To confirm *in-vitro* combination results, CompuSync platform was used in which absorbance was added as input, using individual dose-effect data, software generates possible combination to achieve 50, 75 and 95% effect. It also calculates combination index value (CI) for given combination which help us to understand the possible therapeutic effect of the combination.

	0	A ₁	A ₂	A ₃	A ₄	A ₅
0	0,0	A ₁ ,0	A ₂ ,0	A ₃ ,0	A ₄ ,0	A ₅ ,0
B ₁	0,B ₁	A ₁ ,B ₁	A ₂ ,B ₁	A ₃ ,B ₁	A ₄ ,B ₁	A ₅ ,B ₁
B ₂	0,B ₂	A ₁ ,B ₂	A ₂ ,B ₂	A ₃ ,B ₂	A ₄ ,B ₂	A ₅ ,B ₂
B ₃	0,B ₃	A ₁ ,B ₃	A ₂ ,B ₃	A ₃ ,B ₃	A ₄ ,B ₃	A ₅ ,B ₃
B ₄	0,B ₄	A ₁ ,B ₄	A ₂ ,B ₄	A ₃ ,B ₄	A ₄ ,B ₄	A ₅ ,B ₄
B ₅	0,B ₅	A ₁ ,B ₅	A ₂ ,B ₅	A ₃ ,B ₅	A ₄ ,B ₅	A ₅ ,B ₅

Figure 1: Drug combination setup where symbols A and B represent drug DIC and ASA. The first well served as control and dose 1 was 0.25 times IC_{50} , dose 2 was 0.5 times IC_{50} , dose 3 was IC_{50} , dose 4 was two times IC_{50} and dose 5 was four times IC_{50} . Dose number was represented as subscripts. Total of 25 combinations were tested.

2.5. Statistical analysis of data

Data are presented as Mean $\pm$ SD. The significance of difference among the groups, was assessed using one-way analysis of variance (ANOVA) test followed by Tukey's Honest Significant Difference (HSD) post- hoc test by Graph Pad Prism 7. In figures, symbols represent statistical significance as indicated: 0.12 Non significant (NS), * $p < 0.033$, ** $p < 0.002$ and *** $p < 0.001$.

3. RESULTS AND DISCUSSION

3.1. Docking analysis

The molecular docking approach can be used to model the interaction between a small molecule and a protein at an atomic level, which allows us to characterize the behavior of small molecules in the binding site of target proteins as well as to elucidate fundamental biochemical processes. The docking process involves two basic steps: prediction of the ligand conformation as well as its position and orientation within these sites which is usually referred to as pose and assessment of the binding affinity. AutoDock generate different poses of ligand showing conformations which adapt to interact with its binding site. Poses are ranked as per their binding energy. Binding energy is generally represented as a negative value which indicates that the ligand was bound spontaneously without consuming energy. Binding energy is released when a drug molecule

associates with a target protein, leading to a lowering of overall energy of the protein-ligand complex. Thus, greater the energy released on the binding of a ligand to the protein, greater will be the propensity of the ligand to associate with that protein. Now, to consider the pose hydrogen bonds were calculated. Hydrogen bonds between the protein and new ligand are formed when old hydrogen bonds are broken from nearby water molecules [9]. More the number of hydrogen and less the distance from the proteins means a stronger chance of ligand interacting to the protein. Hydrogen bond having a distance less than 4Å can be considered as possible interaction.

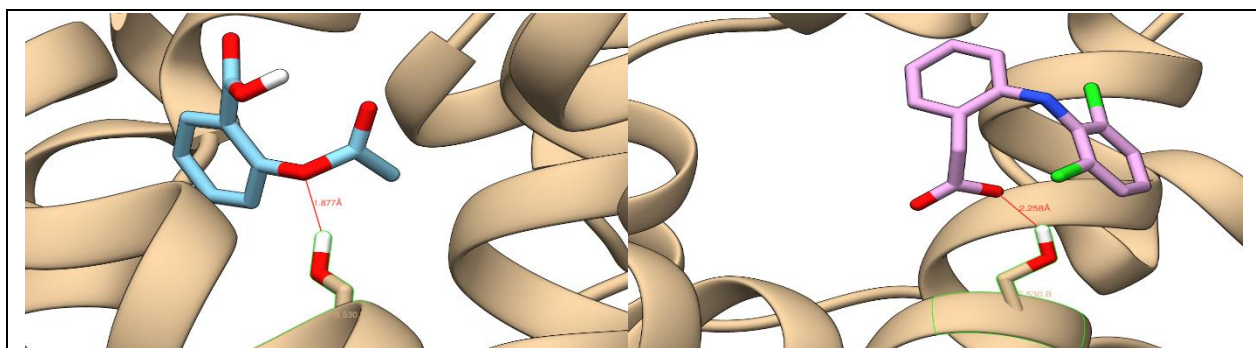


Figure 2: Representative image displaying the binding of ASA and DIC to COX-2 enzyme (Left to Right).

DIC have lower binding energy compared to ASA which indicates it might bind to COX-2 more efficiently and hydrogen bond distance is also comparable. Ser 530 was found to be one of the common residues which show interaction with drug molecule.

3.2. Cell line studies

3.2.1. Cytotoxicity assay

Cytotoxicity tests are based on alterations in permeability of cell plasma membrane and hence the dye is usually absorbed by the dead cells. Alternatively, a metabolic activity can be measured by adding tetrazolium salts to cells. These salts are converted to purple colored formazan product by viable cells that can be measured spectrophotometrically [10]. We attempted to evaluate cytotoxicity of NSAIDs on WRL68 cell line in order to determine the effect of NSAIDs on hepatic cells, as liver serves as a major site for drug metabolism and various biotransformation processes. WRL cells showed growth inhibition in a dose-dependent manner when treated with ASA and DIC at different concentrations for 24 hours. The dose-response curve was constructed for cytotoxicity assessment within the range of 0.1-10mM for ASA and 1-1000µM for DIC. Susceptibility of cells to the drug exposure was characterized by IC₅₀ values. The highest viability of 78% and 77% was observed for DIC and ASA treated cells at 1µM and 0.1mM respectively. IC₅₀ was found to be 220mM and 1.58mM for DIC and ASA respectively. The observed highest cytotoxic activity was found with DIC and ASA at 1000µM and 10mM concentrations respectively. Fig. 3 represents data of percent viability (Mean ±SD) obtained at different concentrations of ASA and DIC after 24 hours. IC₅₀ was calculated using variable slope with four parameter and R square was found to be 0.9078 and 0.919 respectively which indicate good fit.

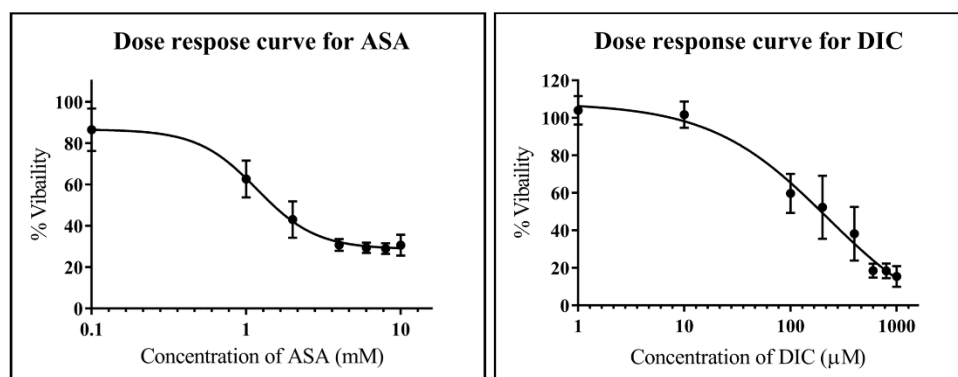


Figure 3: dose response curve for ASA and DIC

As per the report of Blanco et al, IC_{50} for ASA and DIC was found to be 29.3mM and 630 μ M respectively [11]. This difference in IC_{50} can be attributed to the difference in the assay methodology. Elder et al, reported IC_{50} values of salicylates, an active compound of ASA on a different stage of colorectal cancer which was in range of 4 to 7mM, indicating that the higher stage of cancer show greater sensitivity to salicylates [12]. Houseein et al, studies showed IC_{50} for DIC on differentiated HepaRG and undifferentiated HepaRG was found to be 780 μ M and 468 μ M [13].

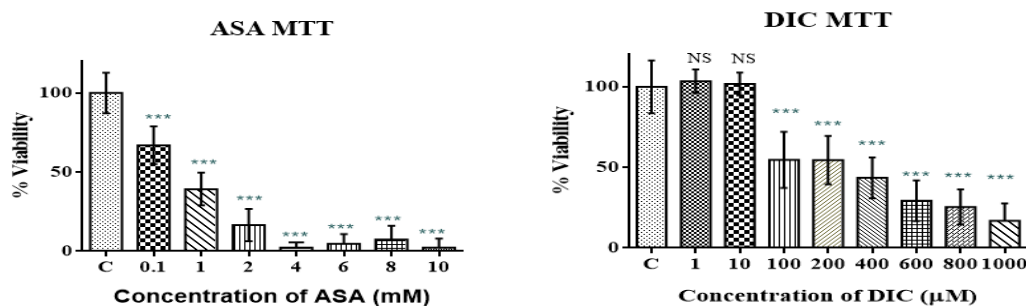


Figure 4: Cell viability of WRL-68 cells against different concentrations of ASA and DIC by MTT assay.

Data represents percentage viability obtained at different concentrations of ASA after 24 hours from Mean $\pm$ S.D. value. In above graph, all concentration were seen to be statistically significant when compared to control. Symbols represent, C:Control, NS:Non Significant and *** p <0.001.

3.2.2.Morphological Assessment Studies

Morphological assessment helps to visualize various event occurring inside the cells. When the cell is committed to an apoptotic pathway, cell membrane begins to form membrane protrusion which is called a “bleb”. This leads to microspikes and on cell fragmentation they are expelled as apoptotic bodies. Formation of vacuoles is suggestive indication of stress also, cells tries to adapt in environment by forming the various elongation. The highly toxic drug causes cell death leading to detachment of cells, forming a circularized floating cell [14]. The cells which were treated with the drugs were then prepared for optical phase contrast microscopy. For ASA treated plates, control plate showed healthy adherent cells. Cells treated with the vehicle also had a normal flattened

appearance for both the plates.

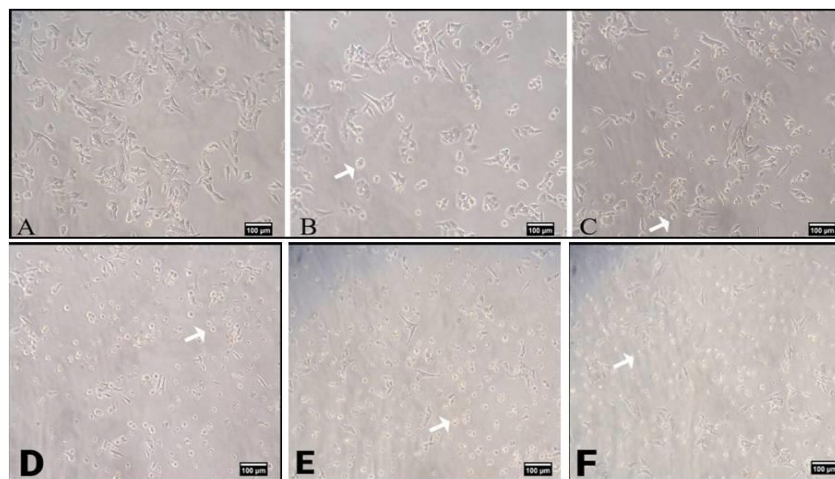


Figure 5.1: Representative photomicrograph showing morphology of WRL-68 cells upon exposure of ASA and imaged by inverted phase contrast microscope (magnification 100X).

A. Untreated cells, B. 0.1mM treated cells, C. 4mM ASA treated cell, D. 6mM ASA treated cells, E. 8mM ASA treated cells, F. 10mM ASA treated cells. Arrow represents the rounding of cells.

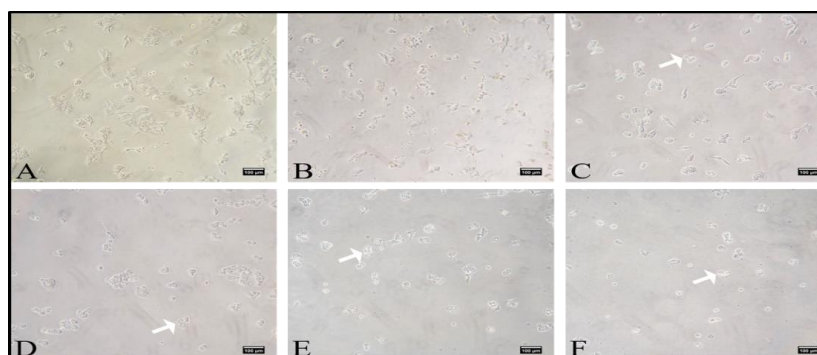


Figure 5.2: Representative photomicrograph showing morphology of WRL-68 cells imaged by inverted phase contrast microscope (magnification 10X).

A. Untreated cells, B. 200µM DIC treated cells, C. 400µM DIC treated cells, D. 600µM DIC treated cells, E. 800µM DIC treated cells, F. 1000µM DIC treated cells. Arrow represents rounding of cells.

However, cells treated with 0.1µM to 2µM ASA, showed progressive characteristic rounding off of the cells (Figure 5.1; B-F), similar results were observed for DIC having the concentration of 200mM to 600mM (Figure 5.2; B-D) and a decline in adherent cell number. Cells underwent marked morphological changes such as reduction in cell volume, cellular shrinkage, granularity. Maximum cells were observed to be floating when treated with 10µM concentration for ASA,(Figure 5.1;F). For DIC the same was observed at 1000mM (Figure 5.2; F) concentration.

3.2.3.Trypan Blue Viability Assay

Trypan Blue assay is based on dye exclusion principle, where dye selectively stains dead cells. Living cells exclude dye, being selectively permeable membrane. A similar fall in the percent cell

viability was observed in the cells treated with ASA and DIC at different concentrations thereby confirming the results obtained via MTT assay. The same concentrations showing cytotoxic potential can be investigated in cancer cell lines for any possible anti-neoplastic properties which can lead to repurposing of these two commonly used drugs.

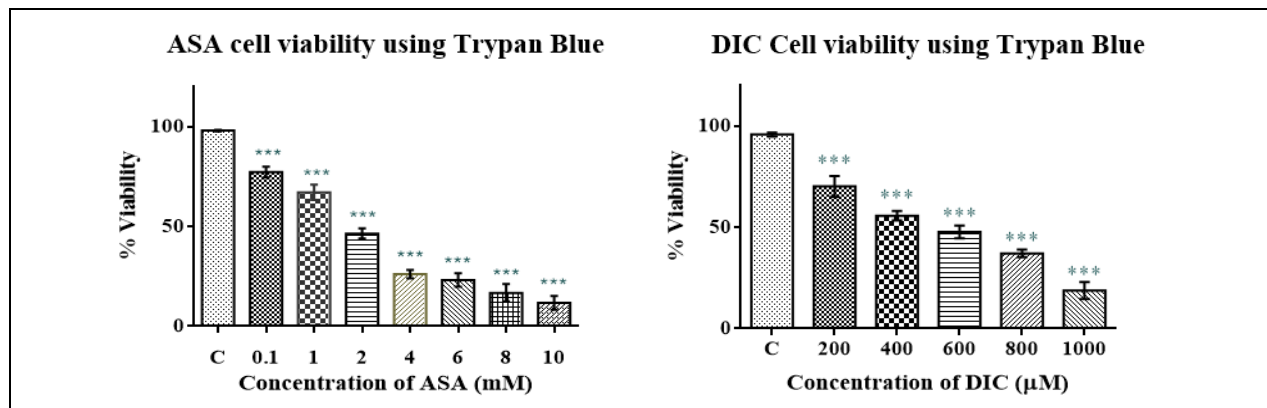


Figure 6: Cell viability of WRL-68 cells against different concentrations of ASA and DIC by Trypan Blue assay.

Data represents percentage viability obtained at different concentrations of ASA and DIC after 24 hours from Mean $\pm$ S.D. value. In above graph, all concentration were seen to be statistically significant when compared to control. Symbols represent, C: Control, NS: Non Significant and *** $p < 0.001$.

3.3. Combinational study analysis

Combination data is analyzed using Loewe additivity and Bliss independent drug interaction models. The Loewe additivity model is defined by equation:

$$\frac{d_x}{D_x} + \frac{d_y}{D_y} = 1$$

Where D_x and D_y are the doses of individual drugs required to exert the same effect as doses d_x and d_y used in combination. If the experimental product of this equation (termed the Loewe combination index) is equal to 1, the data are considered additive; indices of <1 or >1 indicate synergy or antagonism, respectively [15].

The combination study indicates that DIC and ASA are synergistic with CI values ranging from 0.48 to 0.76 as indicated by the Fa-CI table and Fa-CI plot as shown in table 1. The D_m value represents the potency of drug which was found to be 94.62 μ M and 0.523 mM for DIC and ASA respectively. Some combination were synergistic at a lower dose of DIC, whereas the majority of them were the antagonistic which suggest they have common binding sites at COX- 2 enzyme, which was found to be Ser 530 by our *insilico* studies. The synergistic combinations may be considered for further *in-vitro* studies on cancer cell line to reveal any potential anticancer activity and drug repurposing efficiencies. Present methodology of study would help in choosing not only the proper drugs but also the safe combination in which they can be used to amplify their repurposed therapeutic effect.

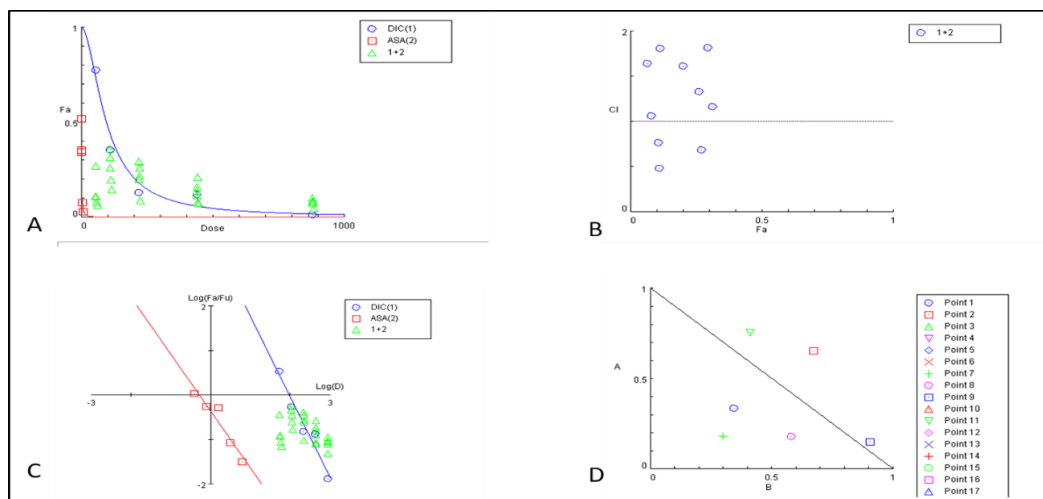


Figure 7: The graphs obtained from the CompuSyn Report for DIC and ASA combinations
A.Dose-effect curves; B. Combination index plot; C. Median-effect plot; D. Isobolograms.

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Insights on OppB Subunit and its Inhibition to Degrade the Growth of *Mycobacterium tuberculosis*

A. NAIR, S. NAGARE*, S. GARSE

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, CBD Belapur, Navi Mumbai-400614, India.

ABSTRACT: Tuberculosis (TB) is counted amongst the fatal diseases like Malaria, AIDS and HIV. *Mycobacterium tuberculosis* and closely related strains of mycobacterium is responsible for this diseased state. Its genome consist of oligopeptide transporters with different subunits. They are considered as nutrient providers in many bacterial pathogens. Their role in *M. tuberculosis* is to provide protection against the host system. As there are lesser studies carried out on the function of these transporters on *M. tuberculosis*. This article performs homology modeling on oppB subunit to generate its structure information. After a refined structure is obtained, its active site analysis is done for the ligand to bind and inhibit the virulent actions. Mutation is also done on the oppB subunit to suggest no change in the interaction of ligand and its active site.

KEYWORDS: Tuberculosis, *Mycobacterium tuberculosis*, oligopeptide transporters, oppB gene

***Corresponding Author: Mr. Sagar Nagare**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: saganagare@gmail.com / sagar.nagare@dypatil.edu

1. INTRODUCTION

The biggest challenge a pharmaceutical industries faced is drug resistance. *M. tuberculosis* is placed amongst one of the infectious pathogen that poses multi-drug resistance [1]. Igen et al 2012 characterized *M. tuberculosis* and its seven sub-species, stating *M. tuberculosis* to be the causative agent [2]. Its genome codes for peptide transporters, belonging to ABC transporter family [3]. The primary function of these transporters is to import or export, the essential compounds that will help in the growth of the bacteria [3, 4]. In *M. tuberculosis*, this transporter system is inclusive of five subunits i.e. Oligopeptide Permease (Opp) A-D and F [4]. Crucially, these subunits are importers. There is very less amount of information and research done on individual functions of these subunits [1, 3]. Therefore, this article focuses on getting an insight of oppB gene by performing comparative modeling using its sequence information. Different shows that Glutathione (GSH) is a perfect ligand that can be used for docking, as it shows bacteriostatic effect [5, 6]. Moreover, there is a comparison of oppB-Glutathione interaction with the mutated oppB gene, to observe whether mutation has any effect on the interaction.

2. MATERIALS AND METHOD

2.1. Protein Structure Modelling and Validation

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From the literature review and getting an understanding of *M. tuberculosis* with its mechanism on human system, oppB gene is selected for our study. The gene sequence is obtained from National Center for Biotechnology Information (NCBI) with [Reference ID: NP_215799.1], which is used as a target to model the 3-D structure by Modeller 9.10. The validation of structure and analysis is done by Swiss PDB Viewer and ProSA-web analysis, which mostly consist of studying energy minimization and Ramachandran plot. Once the structure is modeled, active site is predicted using PPI-PRED. It gives an information of protein-ligand binding to produce the inhibitory action.

2.2. Protein-Ligand Docking and Mutational Analysis

There are 23 analogs of Glutathione created using The Simplified Molecular-Input Line-Entry System (SMILES). The tools used for docking are iGEMDOCK and FlexX. The next is mutational analysis, on the basis of data that is obtained from this process we can determine the proficiency of treating the oppB gene with GSH and thereby finding a novel solution for treating tuberculosis infection. The mutation are first predicted by The Sorting Intolerant from Tolerant (*SIFT*) prediction tool, which takes FASTA sequence of OppB and predicts the possible mutations for each residue. The mutations are achieved about using SPDB viewer's MUTATE option. Furthermore, once the necessary amount of mutations have been generated, the docking process is repeated to check if any difference is observed in the binding of the ligand to the protein active site.

3. RESULTS AND DISCUSSIONS

3.1. Structure Validation

The energy of the modelled protein structure is optimized by SPDB Viewer, and the same structure is used to generate the Ramachandran Plot (Figure 1). It is observed that 87.5 % of the a.a. residues are falling in the favored regions. The ProSA results also suggest that the overall quality of the model lies within the favored range globally and locally.

3.2. Protein-Ligand Docking Results

Out of 23 analogs, highest total energy score is mentioned in Table 1. The docking pose of OppB protein with glutathione analog 38141482 interacts with amino acid Met1, Arg3, Arg7, Arg8, Arg323 and ser325 of the protein active site (Figure 2). The docking pose of OppB protein with glutathione analog 1532230 interacts with amino acid, Met1, Arg7, Arg8, Arg323 (Figure 3).

3.3. Protein-Ligand Docking Results of Mutated protein

In this case, 41 different mutants are developed. Table 2 shows the binding energy of earlier two glutathione analogs after mutation. The mutation analysis shows that there is no significant change in the way the ligand interacts with the active site (Figure 4 and 5). This is due to the fact that these mutations are tolerated by the protein and this does not poses any conformations change that will completely disrupt the molecular structure of the protein.

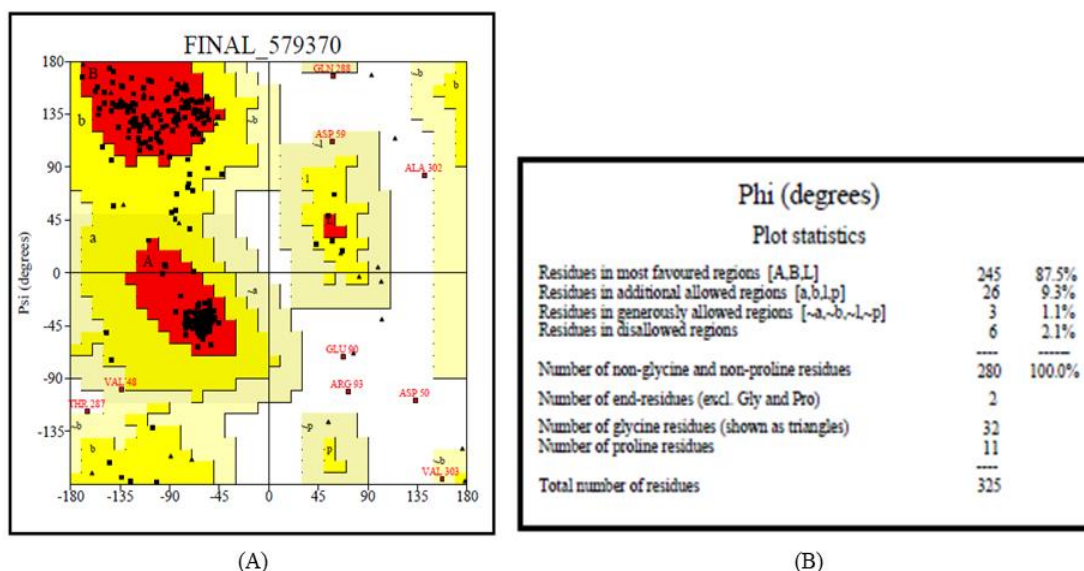


Figure 1: (A) Ramachandran plot for OppB protein. (B) Plot statistics generated by PROCHECK

Ligands	Total Energy	Vander Waal Interaction	H-bond	Electrostatic Energy
38141482.pdb	-122.044	-92.6792	-30.0016	0.636626
1532230.pdb	-102.675	-80.5375	-22.8423	0.704482
3830892.pdb	-101.413	-75.5621	-26.1943	0.343616
4556980.pdb	-95.8772	-62.8842	-33.3259	0.332829
4556979.pdb	-94.1826	-73.3817	-21.8347	1.03376

Table 1: Docking energy of OppB and GSH Analogs

4. DISCUSSION

Various studies have experimentally explained the functions of oppA and oppD. Yet, there is a need to focus on the other subunits. The result obtained suggests that, an oligopeptide transporter molecule like oppB gene product when inhibited using a glutathione ligand molecule, proves to help in the degradation in the growth of mycobacterium macrophages.

5. CONCLUSION

This current study of the protein structure provides clarity on how this vital protein could be manipulated in order to modify its function. The overall objective is to study the effects of mutations on the *M. tuberculosis* protein and its active sites. Though, this article work is entirely computer/bioinformatics proof, working it out in a wet lab environment could prove a challenging task.

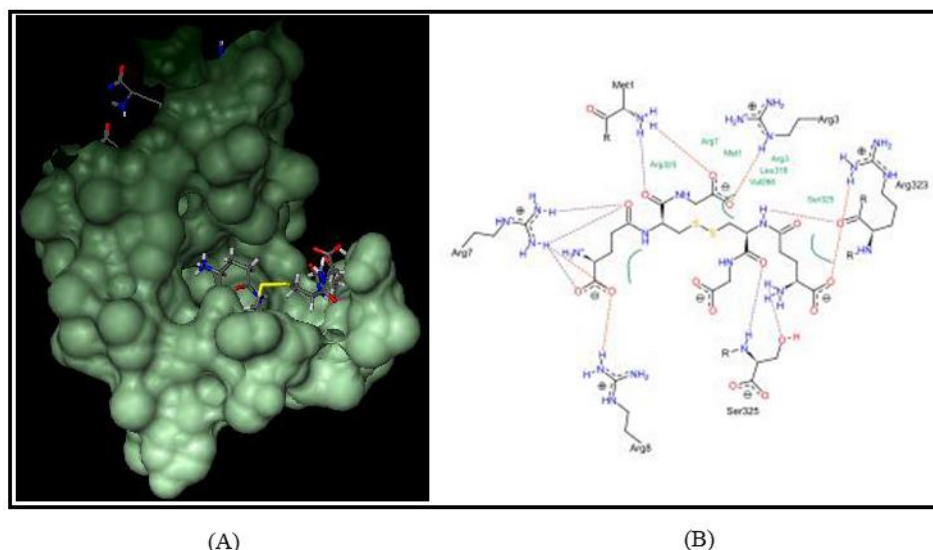


Figure 2: (A) Docking pose of OppB protein with glutathione analog 38141482. (B) Bond level interaction of the same.

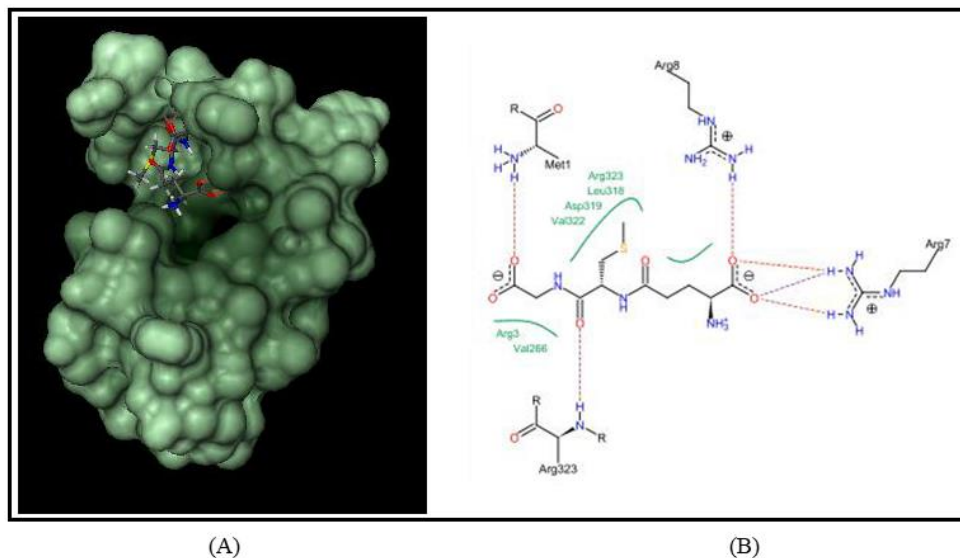


Figure 3: (A) Docking pose of OppB protein with glutathione analog 1532230. (B) Bond level interaction of the same.

Ligands	Total Energy	Vander Waal Interaction	H-bond	Electrostatic Energy
38141482.pdb	-122.044	-92.6792	-30.0016	0.636626
1532230.pdb	-102.675	-80.5375	-22.8423	0.704482

Table 2: Docking energy of mutated OppB and GSH Analogs

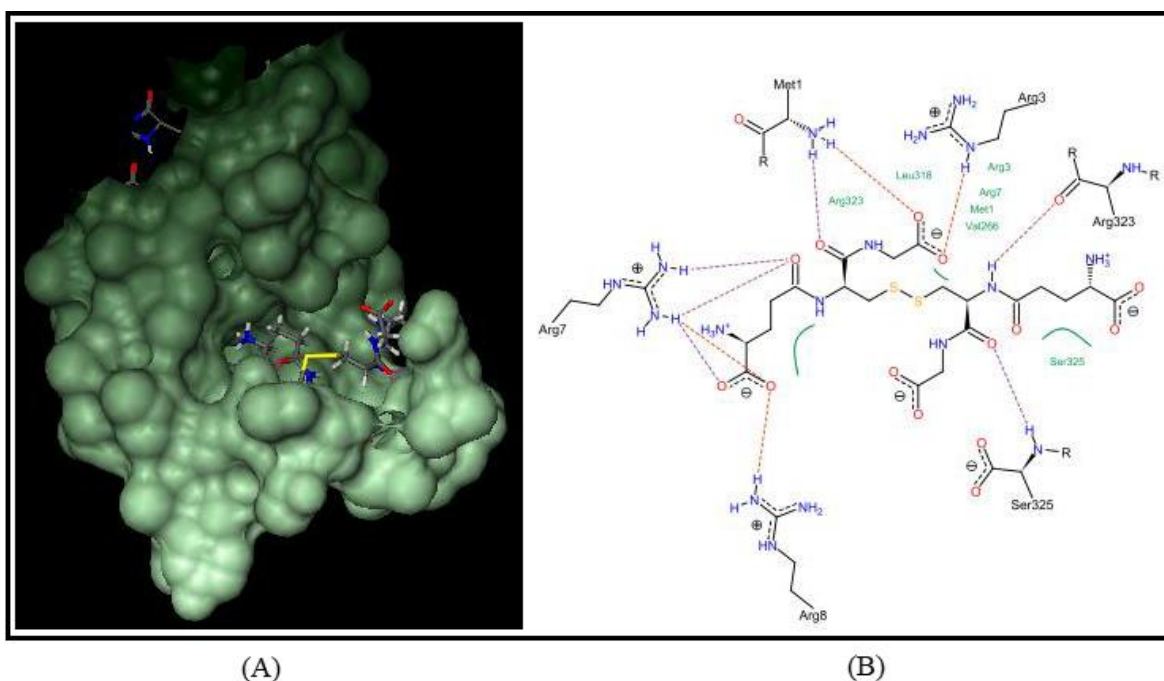


Figure 4: (A) Docking pose of mutated OppB protein with Glutathione analog 38141482. (B) Bond level interaction of the same.

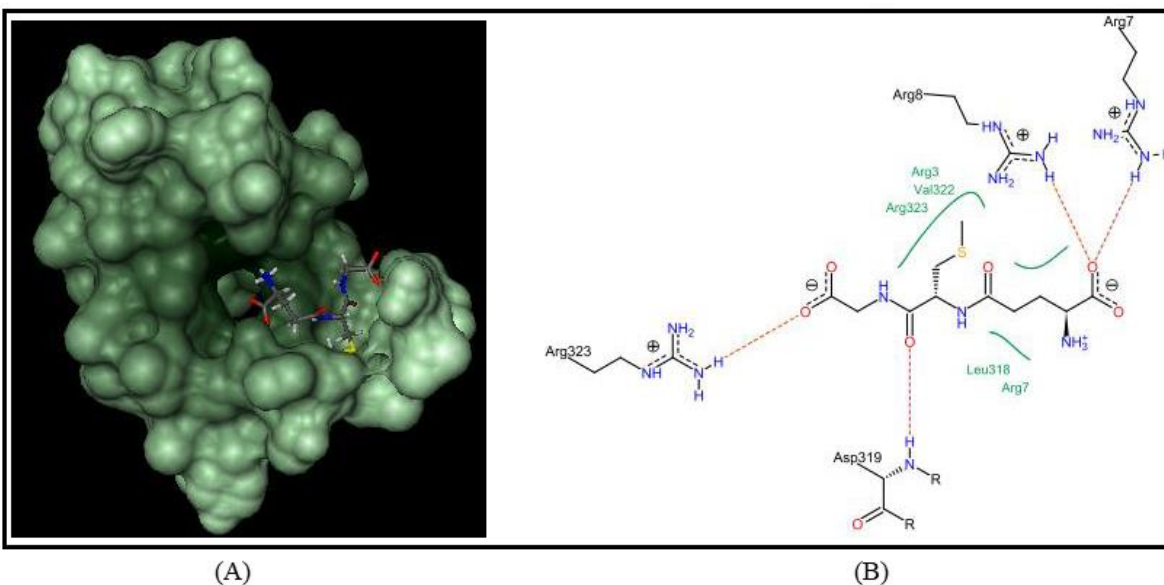


Figure 5: (A) Docking pose of mutated OppB protein with Glutathione analog 1532230. (B) Bond level interaction of the same.

6. ACKNOWLEDGEMENT

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7. CONFLICT OF INTEREST

We have no conflicts of interest to disclose.

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Preparation and Characterization of Chitosan Nanoparticles Functionalized with Plant Extract for Antibacterial Activity against *Escherichia coli* and *Staphylococcus aureus*

N. JOSHI, R. GOKARN, D. GARG*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No. 50, Sector 15, CBD Belapur, Navi Mumbai-400614, India.

ABSTRACT: The present study was aimed at synthesis and evaluation of antibacterial activity of chitosan nanoparticles functionalized with plant extracts. Nanoparticles were synthesized using ion gelation technique. A ratio 1:3 of chitosan: TPP yielded best nanoparticle of the size ranging from 300-400 nm as per the DLS method. A zeta potential value of 22.84mV indicated the stability of nanoparticles. The nanoparticles were further characterized and confirmed by SEM and TEM. Synthesis of nanoparticles functionalized with extract of *Psidium guajava* and *Mentha piperita* was carried out. Compared to chitosan alone its nanoparticles showed higher antibacterial activity as indicated by larger zone of inhibition against both *E. coli* and *S. aureus*. However, chitosan nanoparticles functionalized with plant extract showed lower antibacterial activity. The methanol extract of *M. piperita* showed antibacterial activity against both the bacterial test organisms. Both aqueous and methanolic extracts of *P. guajava* showed antibacterial activity against only *S. aureus*. The results demonstrated the need to further optimize preparation of functionalized nanoparticles in order to utilize it appropriately. The present study was aimed at synthesis and evaluation of antibacterial activity of chitosan nanoparticles functionalized with plant extracts. Nanoparticles were synthesized using ion gelation technique. A ratio 1:3 of chitosan: TPP yielded best nanoparticle of the size ranging from 300-400 nm as per the DLS method. A zeta potential value of 22.84mV indicated the stability of nanoparticles. The nanoparticles were further characterized and confirmed by SEM and TEM. Synthesis of nanoparticles functionalized with extract of *Psidium guajava* and *Mentha piperita* was carried out. Compared to chitosan alone its nanoparticles showed higher antibacterial activity as indicated by larger zone of inhibition against both *E. coli* and *S. aureus*. However, chitosan nanoparticles functionalized with plant extract showed lower antibacterial activity. The methanol extract of *M. piperita* showed antibacterial activity against both the bacterial test organisms. Both aqueous and methanolic extracts of *P. guajava* showed antibacterial activity against only *S. aureus*. The results demonstrated the need to further optimize preparation of functionalized nanoparticles in order to utilize it appropriately.

KEYWORDS: Antibacterial activity, Chitosan, ion gelation, nanoparticles, plant extract

***Corresponding Author: Dr. Deepa Garg**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: deepa.garg@dyatil.edu, Phone number: +91 22-27563600

Mobile: +91 9920443369

1. INTRODUCTION

One of the most important application of nanoparticle technology is in non invasive drug delivery which offers opportunities for controlled release, protection of active components from enzymatic or environmental degradation and localized retention. Out of various nanoparticle drug delivery methods, polymeric nanoparticles have received significant attention as they are biodegradable, biocompatible and their preparation methods are convenient. Chitosan-based NP are well known for their low toxicity, mucoadhesion, tunable physical properties and have been reported to use for drug delivery to treat infections of various organs like brain, eyes etc.

Chitosan is an important derivative of chitin, produced by removing the acetate moiety from chitin. Chitosan is a polymer of glucosamine and N-acetyl-glucosamine linked by a beta 1-4 glycosides bond. It is derived from shells of crustaceans like prawns or crabs, as well as from the cell walls of fungi. It is a biopolysaccharide, cationic, highly basic, mucoadhesive and approved by the U.S. FDA for tissue engineering and drug delivery. Chitosan nanoparticles have been produced through various methods like emulsification, ionic gelation method [1] reverse micellar method, self-assembling method [2, 3,] etc. Recently green synthesis of nanoparticles have received much attention as an alternative to chemical synthesis. In this regard extracts of various plant species have been used for example *Bischofia javanica* L., *Daucus carota*, *Solanum lycopersicum*, *Hibiscus*, *Moringa oliefera* flower, *Bacopa monnieri*, *Citrus unshiu* peel, *Cymbopogon flexuosus*, the plant extract of Aloe Vera leaves [4]. Plant extracts contain various phytochemicals that may act both as reducing agents and stabilizing agents in the synthesis of nanoparticles. The synthesis of nanoparticles using plant extract provides advancement over other methods as it is simple, one step, cost-effective, environment friendly and relatively reproducible [4].

The present study was conducted to optimize the synthesis of chitosan nanoparticles and the antimicrobial evaluation of extracts of *Psidium guajava* and *Mentha piperita* loaded with chitosan nanoparticles.

2. MATERIAL AND METHODS

2.1.Collection of Plant material and Extract Preparation

The fresh and tender guava and mint leaves were collected from a local garden. The authentication of plant leaves was done at the Blatter Herbarium, St. Xaviers College, Mumbai. The leaves were thoroughly washed, shade dried and then crushed by electric grinder. 30 gm of leaves powder subjected to soxhlet extraction. The extracted solutions were concentrated in a rotary evaporator and stored at 4 °C for further use. Glycerol stock of *S. aureus* and *E.coli* was obtained from School of Biotechnology and Bioinformatics, D. Y. Patil University.

2.2.Preparation of Chitosan Nanoparticles

Ionic gelation method [5] was used for chitosan nanoparticles preparation. 0.4% (w/v) chitosan solution in 1% acetic acid and aqueous solution of 0.25% (w/v) of TPP were prepared. To the chitosan solution TPP solution in different ratio (4:1, 3:1, 2:1, 1:1) was added drop-wise under constant stirring. The mixture was stirred for 6 hours and was centrifuged at 4°C with a speed of

9000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with distilled water to remove excess TPP and chitosan.

2.3.Synthesis of Chitosan nanoparticles functionalized with plant extract

The mixture of 0.5% methanolic plant extract and 1% chitosan was stirred overnight to allow for the incorporation of the plant extract into the chitosan before the synthesis of the nanoparticles. The 1.5% TPP solution was added drop-wise to the chitosan – plant extract solution while under constant stirring and centrifuged at 4°C with a speed of 9000 rpm for 15 minutes [6]. The supernatant and pellet were stored.

2.4.Antibacterial activity assay

Antibacterial assay for various samples were checked by well- diffusion method. Nutrient agar plates were inoculated separately with *E.coli* and *S. aureus* using the spread plate technique (1×10^8 cfu/ml). For checking the antibacterial activity chitosan nanoparticles functionalized. For this purpose supernatant and pellet (described above) were used. Streptomycin and itraconazole were used as positive control (PC) for antibacterial and antifungal activity assay respectively. 1% acetic acid was used as negative control (NC). After 24 hrs at 37 °C incubation, the plates were observed for the results and zone of inhibition measured. The experiment was done in triplicate and values are presented as Mean $\pm$ S.D.

3. RESULTS AND DISCUSSION

3.1.Synthesis of chitosan nanoparticles and CNP loaded with plant extracts

The chitosan nanoparticles are formed upon addition of TPP solution to CS solution under constant magnetic stirring and depending on the ionic interaction of CS with TPP, it gradually leads to the low aqueous solubility of CS. It has been found that this CS/TPP ratio is decisive and the size characteristics and biological activities of chitosan are affected by it [7, 8, 9]. The size characteristics of different CS/TPP ratio combinations and found sample no. 2 to be within the optimum range for the nanoparticles.

Sample no.	Chitosan (0.4%) ml	TPP (0.25%) Ml	CS/TPP (w/w) ratio	Opacity
1	17.5	7	4:1	Clear
2	17.5	10	3:1	Opaque
3	17.5	13	2:1	Opaque
4	17.5	28	1:1	Cloudy

Table 1: Effect of Ratio of Tpp: Chitosan on Synthesis of Nanoparticle

3.2.Characterization of Chitosan nanoparticles

According to TEM the images, bare chitosan nanoparticles were spherical and had smooth surfaces

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(Fig 1). The average diameter of the dried particles was 42.95nm.

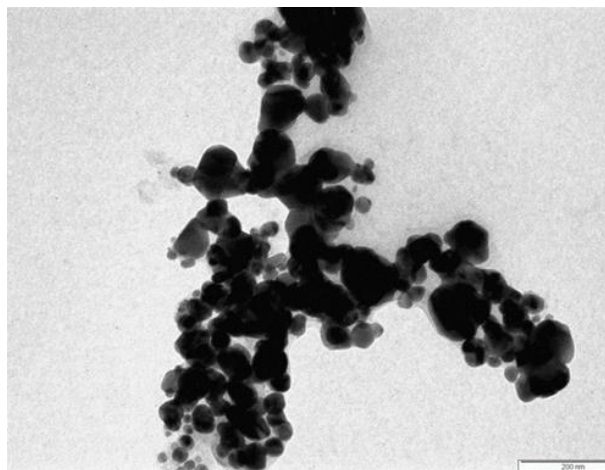


Figure1: TEM image of chitosan nanoparticles

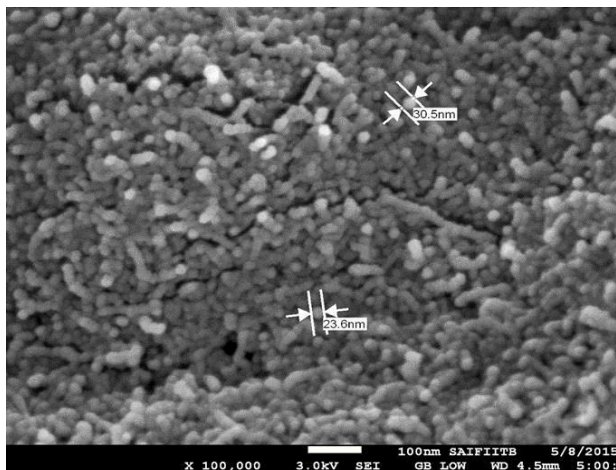


Figure2: SEM image of chitosan nanoparticles

The SEM images indicated the formation of irregularly shaped particles with some agglomeration. The agglomeration of nanoparticles constitutes the micro-particles which may decrease the capability of nanoparticles to penetrate the cellular structure. So, no size related toxic effect was expected. The size of the nanoparticles ranged from 20-30nm. Our results were in consistence with those reported by Liu and Gao [5].

3.3. Dynamic Light Scattering (DLS)

The DLS technique was used to analyze the particle size distribution. DLS technique determined the hydrodynamic size of a particle but it is capable to determine the size or size distribution only when the sample particles are dispersed in some liquid, in this case deionized water. Chitosan nanoparticles recorded by DLS had an average diameter of 329nm (Fig. 3). The reason for the larger diameter size could be due to particle swelling and/or aggregation when dispersed in deionized water [5].

3.4. Zeta Potential

Zeta potential of nanoparticles is an indirect measure of the surface charge. This potential analyze the colloidal stability of nanoparticles. The chitosan nanoparticles are characterized with high zeta potentials and are attracted to negative surfaces such as cell membranes. Chitosan nanoparticles have an average zeta potential of about 22.84mV and this may be due to the presence of amino groups in the chitosan chain [5]. In the formation of chitosan nanoparticle, positive surface charge is important as it is said to prevent particles aggregation and help in the attachment to membranes via electrostatic interaction.

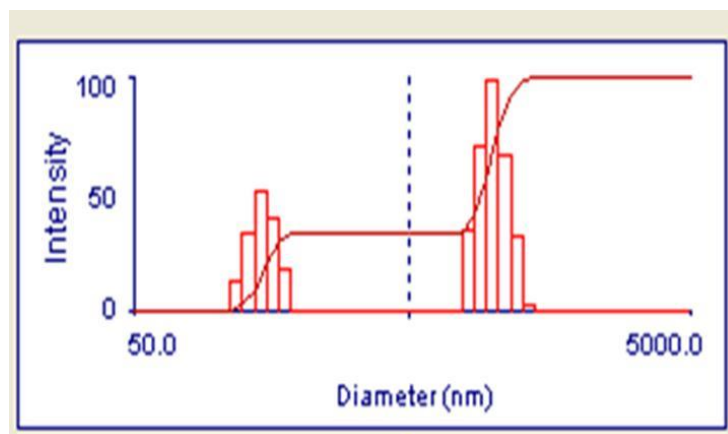


Figure 3: Dynamic light scattering (dls) image showing the size distribution of chitosan nanoparticles

3.5. Antimicrobial activity of chitosan, chitosan nanoparticles (CS-NP) and CS-NP functionalized with plant extract

Antimicrobial activity of chitosan increased with concentration indicating that the concentration of chitosan did influence the antimicrobial effect. As shown in the Table 2, chitosan inhibited the growth of all three test microorganisms. Chitosan solution (0.4%) exhibited antimicrobial effect against all three microbial isolates tested. CS showed maximum antimicrobial activity against *E. coli* (22.6 ± 3.05) and lowest activity against *A. niger* (13.3 ± 3.05). Out of the two bacterial cultures, the Gram negative organism, *E. coli* was more sensitive to chitosan than the Gram positive *S. aureus* [10, 11, 12,].

Sr. No.	Test Microorganisms	Zone of Inhibition diameter (mm)		
		CS	PC	NC
1	<i>Escherichia coli</i>	22.6 ± 3.05	26.6 ± 3.05	0.0
2	<i>Staphylococcus aureus</i>	20.6 ± 3.05	24.6 ± 3.05	0.0
3	<i>Aspergillus niger</i>	13.3 ± 3.05	19.3 ± 1.52	0.0

Table 2: Zone of inhibition by 0.4% chitosan solutions (50-150 $\mu\text{g/ml}$) against *E. coli* and *S. aureus*.

CS: Chitosan, PC: Positive control, NC: Negative control

Chitosan nanoparticle synthesized in the 3:1 CS-TPP ratio showed highest antimicrobial activity with respect to other ratios indicating that this particular ratio of CS-TPP has improved cross-linking configuration.

CS-NPs showed maximum antimicrobial activity against *E. coli* (13.6 ± 2.08) and lowest activity against *A. niger* (13.0 ± 1.41) (Table 3). Within the two bacterial cultures, the Gram negative bacteria, *E. coli* was more sensitive to CS-NPs than the Gram positive bacteria *S. aureus*.

The chitosan nanoparticles functionalisation was carried with only methanol extract of *Mentha piperita* since antibacterial and antifungal activity was exhibited by it. The chitosan nanoparticles with extract was slightly greener in colour as compared to the chitosan solution. The appearance of colour indicated that the nanoparticles were functionalized with plant extract. The results of antimicrobial assay for the CS-NPs functionalized plant extracts (Table 4) showed that both plant extract and supernatant inhibited the growth of both the bacterial cultures whereas no antifungal activity was seen. The supernatant of the functionalized plant extract showed low antibacterial activity as with that of the pellet showed no antimicrobial activity. Our results are in accordance with other studies also [13, 14].

Sr. No.	Test Microorganisms	zone of Inhibition diameter (mm)		
		CS-NP	PC	NC
1	<i>Escherichia coli</i>	13.6±2.08	26.6±3.05	0
2	<i>Staphylococcus aureus</i>	12.0±1.0	24.6±3.05	0
3	<i>Aspergillus niger</i>	13.0±1.41	19.3±1.52	0

Table 3: Zone Of Inhibition Shown By Chitosan Nanoparticles

CS-NP: Chitosan nanoparticle, PC: Positive control, NC: Negative control

Sr. No.	Methanol extract of <i>M. piperita</i>	Zone of inhibition (mm)		
		<i>E. coli</i>	<i>S. aureus</i>	<i>A.niger</i>
1	PE	4	8	-
2	Supernatant	3	6	-
3	Pellet	-	-	-

Table 4: Zone Of Inhibition by Functionalised Cs-Nps

PE: Plant extract

4. CONCLUSION

The nanoparticles obtained in the present study by ion gelation method have small particle size with an average size of 42.95 nm according to the Transmission electron microscopy (TEM). The chitosan nanoparticles functionalized showed very low antibacterial activity which could be due to insufficient concentration of loading extract sample or the size characteristics or the stability of physicochemical properties of nanoparticles.

5. ACKNOWLEDGEMENT

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Microbial Pigments as Alternative Source for Bio-Colorants

T. ROY¹, S. LILWANI², J R. PARVATHI^{3*}

¹School of Environmental & Biological Sciences, Rutgers University, New Brunswick, New Jersey 08901.

²School of Biotechnology & Bioinformatics, D Y Patil Deemed to be University, Navi Mumbai Maharashtra 40076.

³Somaiya Initiatives for Research & Consultancy (SIRAC) Somaiya Vidyavihar, Vidyavihar (E) Mumbai, Maharashtra 400022.

ABSTRACT: The potential of microbes is numerous, from being an antibiotic producer to recombinant host, enabling bio-remediation to the utility as bio-battery. Another application characteristic to a certain type of microbes, the pigmented ones, is its usability as bio-colors. Even though plant pigments are considered as the quintessential bio-color, their seasonal dependability for growth, bulk requirement and laborious work for pigment production have proved detrimental for large-scale production. These limitations can be circumvented with microbes that have no seasonal preferences and require only a minimal seed concentration for a mass production when incubated at optimal growth conditions. The current study was carried out to explore the appositeness of dark yellowish pigmented microbe (Y_01) obtained from a soil sample near seawater for its pertinence as coloring agents. Owing to its niche, artificial seawater medium was the preferred growth medium for Y_01, incubation at 28⁰C under dark conditions was found optimal for the growth. Among the various solvents used for extraction, with ethanol Y_01 gave maximum pigment extraction. With rota-vaporization of the ethanol extract 0.51gm/L of powdered pigment was obtained whereas with crude extraction method 9gm/L was obtained. In comparison to natural mordants, metallic mordants roved as better fixing agents of which ferrous sulphate showed the highest light fastness properties. Successful trials employing microbial pigments as colorants for candles, paper, and bioplastics provided a holistic conclusion to the objective.

KEYWORDS: Pigmented bacteria, Biocolors, Synthetic colors, Pigment solubility, Mordants

***Corresponding Author: Dr Parvathi J R.**

Somaiya Initiatives for Research & Consultancy (SIRAC) Somaiya Vidyavihar, Vidyavihar (E) Mumbai, Maharashtra 400022.

Email Address: parvathi.jr@somaiya.edu

1. INTRODUCTION

Color has evolved much beyond from its definition as a ‘property possessed by any object that tenders diverse sensations on the eye as a result of the way it reflects or emits light’ to that of an identity. Rather than a physical entity, color now stands to reflect ones mood or personality in physiological studies or as an indicator to mark freshness of food in quality management or as the best choice of branding tool in fashion and/or marketing fields. In spite of these wide range of applications, the health hazardous effects of synthetic dyes [1, 2] have now caused the resurgence

of age old methods and natural dyes as potential source. Dyeing practices with natural colors or biocolors [3] are evident in archaeological finding that dates back from to ruins of Indus Valley Civilization (2600 BC -1900 BC) to that of Bronze - Iron Age [4, 5]. The major sources of natural colors were predominantly plants followed by insects & minerals [6]. Almost all the parts of the plants from roots to leaves and even flowers were used for dye extraction [7] which normally involved a lengthy process starting with boiling the plant material for hours to obtain the dye. The fabric that need to be colored were then immersed in the dye solution that were either heated or allowed to stay for days to weeks to get the desired colour. Not all dyes immediately color the fabric and if they do so, fade with subsequent wash. Mordants are fixatives that are required to bind these natural colors on the fabric, the standardisation of the different types of mordants against individual dyes should be done for obtaining good results [8]. Even though metallic mordants of chromium, copper, iron, and tin are commonly used they are toxic and irritant causing environmental hazards [9].

Depending solely on plants as the biocolor source is detrimental owing to the seasonal dependence, bulk requirement and the laborious practice involving traditional knowledge and know-how of extracting natural dyes from plant source. Another alternative for natural colorants are microbial pigments [6]. Apart from being physical parameter for microbial identification, pigmentation plays an apparent role in photosynthesis [10], UV protection [11], & virulence [12]. These microbial pigments have found their applications as antioxidants [13], antimicrobial agents [14] as well as textile dyes [5]. This pilot study was intended to explore the possible application of microbial pigment as bio colorants on different fabrics (with and without mordants), paper, wax and bioplastics. Based on the rationale, a pigmented organism from soil sample was isolated followed by partial optimization of the growth condition to elute the pigments for the mentioned applications.

2. MATERIALS AND METHODS

2.1.Bacterial isolation and growth conditions

Soil samples were collected 3 cm deep of O or A horizon (topsoil) from the sea shore of Girgaon Chowpatty, Mumbai [18°57'17.6"N 72°48'35.2"E]. Following serial dilution, the samples were streaked on seawater agar to mimic the niche of the sample source. Only yellow coloured pigmented microbes were obtained, of which the darkest yellow pigmented isolate (Y_01) was taken for further study. A set of trials were carried out to standardize & maximize pigment retrieval only with respect to varying temperature of incubation (16°C, 28°C, 37°C & 45°C) and incubation period (12, 24, 36, 48, 60 & 72 hours). Preference for light and dark as well as cold and room temperature were assessed for increased pigmentation.

2.2.Extraction and Characterization of pigment

Dual trials of abstraction were attempted for pigment extraction, one employing solvents (solvent extraction) and the other without solvents (crude extraction). As a prelude to both solvent and crude extraction, bacterial cell mass obtained from overnight grown bacterial culture were used. The culture was centrifuged at 10,000 rpm for 10 min for sedimentation, supernatant was discarded and pellet used as the starting material. For the solvent extraction, a battery of solvents (Methanol,

Acetic acid, Acetone, Ethyl acetate, Hexane, Dichloromethane, Petroleum ether) with varying range of polarity were studied. The pellet was suspended in respective solvents; this step was coupled with sonication at various time intervals (10-20min) to assess whether this physical method accentuates extraction. After choosing the ideal solvent, the extracted pigment were reduced to 5% of original volume using rota-vapour and oven-dried in open glass petri plates for 2 days at 40°C. The powder obtained were collected and stored in brown bottles. For the crude extraction, centrifuge tube with pellets were subjected to autoclaving procedure twice to heat kill the bacteria. After overnight oven-drying to remove traces of medium, the dead cell mass was scraped out with wooden stick and dispensed into a bottles for storage. The extracted pigment using solvent extraction was analysed with UV-Visible spectrophotometer for detecting the absorption maximum (λ max). To analyse the identity and purity of the pigment, thin layer chromatography was carried out with ethyl acetate and acetone (7:3) as the solvent system.

2.3.Reaction of pigment with acid, bases and mordants

Powdered pigments (0.5gms) obtained after rota-evaporation were treated with few drops of 12N HCl, 5N NaOH and 20% KOH on glass slide. The pigment obtained via solvent extraction procedure was dissolved in different mordants/solvents of 100% concentration (salt water, acetic acid, citric acid, sodium carbonate, ferrous sulphate, copper sulphate and thiourea) in test tubes and recorded for color changes.

2.4.Application of pigment

2.4.1.Application as fabric colorants

A representative fibre, each of plant (cotton), animal (woollen) and synthetic (nylon) origin were selected for dye application. For this purpose, 3.5cm x 2.5cm sized pieces of cotton and nylon fabrics & 12cm woollen thread knotted in a loop were taken. For each fabric, two sets were prepared, one was degummed and bleached whereas the other wasn't. Degumming was carried out by treating the cut fabrics with 15g/L soap solution, which was later immersed in water bath (80°C) for 60min. After thorough washing, bleaching was carried out by soaking the fabrics in hydrogen peroxide (3g/L) keeping the incubation time and temperature same as before. Both the fabrics were thoroughly washed with water and dried. Simultaneous mordanting procedure was carried where in dye and mordant were applied together to fabrics. A series of natural (salt water, acetic acid, citric acid, sodium carbonate) and metallic mordants (ferrous sulphate, copper sulphate & thiourea) were tried. The fabrics were soaked with the extracted pigment & respective mordant in a water bath (80°C) for 30min, dried & washed with water to remove any excess dye and mordants. The fabrics were tested for color fastness in two ways, one set of each were exposed to sunlight (light fastness) and the other was subjected to washing and simultaneous drying (wash fastness). Both the test were assessed for a period of 7 days, undyed fabric were taken as a control for comparison.

2.4.2.Application as paper colorants

White A4 size paper was cut into circle of 3.5cm diameter and dipped into the different dye baths and was allowed to stand for 30 minutes. Post dyeing, the paper was carefully removed from the

flasks and blow dried using hot air blower. The paper discs were subjected to light exposure test for 7 consecutive days to check for its color fastness properties.

2.4.3. Application as candle colorants

1gm powdered pigment mixed with 3ml of the selected solvent was added to 7mL molten candle wax, obtained by recycling old white candles. The mixture was allowed to solidify under room temperature with a candle wick in the centre.

2.4.4. Application as bioplastics colorants

Bio-plastic preparation involved use of potato starch and its polymerization using food grade glycerol as plasticizer. Starch was prepared from peeled potatoes (400gm) that were cut into pieces and mashed in 100mL water using grinder. The mashed potato was strained using muslin cloth and the extract was collected in a 500mL beaker. The process was repeated 3-4 times & the collected extract was allowed to stand still for the starch sedimentation. The white crude starch obtained was repeatedly washed with distilled water and oven dried in an open petri plate for 3hrs at 50°C. 5gms of the extracted starch was mixed with 50mL distilled water, 6mL of 0.1M HCL & 4mL glycerol for preparing bioplastic. The mixture was subjected to heat with occasional stirring to avoid any lumps formation, before gelation the extracted dye was added. The biolistic mixture was poured into petri plates & oven dried overnight at 40°C. Bioplastic prepared without adding of extracted pigment was considered as control.

3. RESULTS & DISCUSSION:

3.1. Bacterial isolation and growth conditions

Y_01 grown on seawater agar medium showed growth at 28°C and even more at 37°C [Fig 1] with no special preference of dark or light conditions. Even though the microbial growth was observed within 12 hrs of incubation, the appearance for pigmentation was evident after 24hrs which surged maximum at 72hrs and not beyond that. Increased pigmentation and bigger colonies were observed in petriplates after storing the grown cultures under cold conditions (4°C).

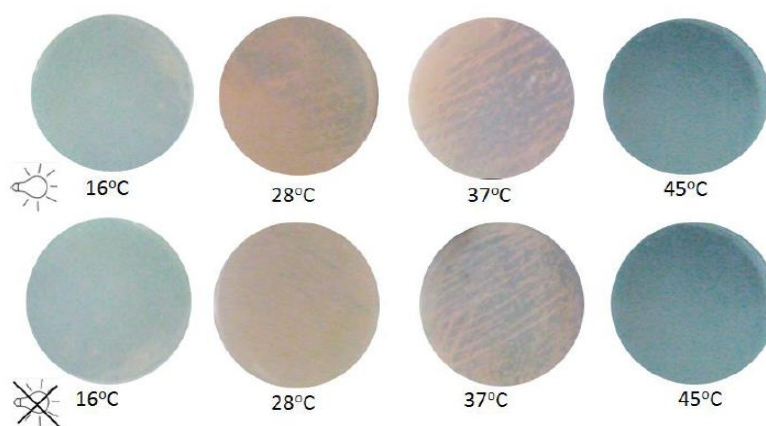


Figure 1: Growth of Y_01 at different temperatures at both light (above) and dark conditions

3.2.Extraction and Characterization of pigment

Across the various solvents tested, maximum solubility of the pigment was observed in ethanol followed by methanol and then acetic acid & acetone. Partial solubility was observed in ethylacetate whereas pigment showed insolubility in hexane, dichloromethane and petroleum ether. Coupling of the solvent extraction with sonication showed maximum extraction at 20min, on and after this stipulated time the result were the same. Solvent extraction with sonication was found be more effective as retrieval time was 50% more than solvent extraction alone. Ethanolic pigment extract of Y-01 showed maximum absorbance at 450nm with an R_f value of 0.891 in TLC using ethyl acetate and acetone as solvent system. The result indicates that the pigment is of carotenoid group but the exact class of carotenoid can be confirmed only after structural assays that is beyond the scope of this study.

3.3.Reaction of pigment with acid, bases and mordants

Using rota-vaporation of ethanolic pigment extract, 0.51gm/L of powdered pigment was obtained whereas crude extraction provided 9gm/L of the same. Dark yellowish colored pigment changed to brown in contact with 12N HCl, no change was recorded with 20% KOH and 5N NaOH.

The extent of coloration of the pigment on the surface or any material are influenced by mordants which form coordination complexes with dyes attaching it firmly to the fabric [15]. Tannins, oil and metallic mordants are generally classes of mordants used [16] of which metallic one are most preferred owing to its strength in binding to the fabric and the colouration imparted to the fabric.. The complex formation of the dye with the metallic component of the mordant not only strengthens the dyestuff fixation but also changes the colour of dyeing [8]. On mixing the natural and metallic mordants individually with pigment extracts in equal amount, the colour change observed [Fig 2].



Figure 2: Coloration of mordant with and without microbial pigment

3.4.Application of pigment

3.4.1.Application as fabric colorants

All three fabrics (cotton, woollen and nylon) showed similar result with and without degumming treatment. Among the mordants tried, natural mordants showed least resistance to fading and colour removal during exposure to light and washing. Both metallic mordants helped in fixing the pigments and dyeing the fabrics, ferrous sulphate showed more lightfastness and wash resistance than copper sulphate in all the fabrics [Fig 3, Fig 4, Fig 5].

3.4.2.Application as paper colorants

With respect to paper dyeing, salt water & sodium carbonate showed negligible dyeing. Both copper sulphate and ferrous sulphate showed effective coloration on paper and held the colour even after 7 days of exposure to sun [Fig 6].

3.4.3.Application as candle and bioplastic colorants

Applicability of microbial pigments were successfully shown in bioplastic [Fig 7a & b] as well as in candles (Fig 7c & d) with the halo of the candle light bearing an orange flame.

4. CONCLUSION

The current study was only intended to explore the possibility of using microbial pigments as alternative source of biocolors. Both crude and solvent extraction were efficient in pigment extraction, with former being a fastidious process & latter being the best scenario for preparing dye stuff. Light as a physical parameter did not appear to induce increased growth but cold conditions showed augmentation of colony size and pigmentation. A similar study on other pigments need to be carried out to comment on any possible link between cold conditions and pigmentation. Comparative study of metallic and natural mordants indicated metallic mordants having better fixing capacity and light fastness property. A successful application as colorants were proved with fabrics, candle and bioplastics but other facets involving its utility as cosmetic and food colorants are intended for future. An in-depth study on the particle size, solubility of the pigment, interaction of mordants as well understanding the biological property and structural characterisation of pigment can throw more light and channelize the train of thought for customised applications. Antioxidant and antimicrobial property of microbial pigments could be reconnoitred for pharmaceutical sector also.

5. ACKNOWLEDGMENT

The work was carried out in School of Biotechnology & Bioinformatics, D Y Patil (Deemed to be) University, Navi Mumbai.



Figure 3: Results of dyed cotton fabric and exposure of light (left) & washing (right) for consecutive 7 days

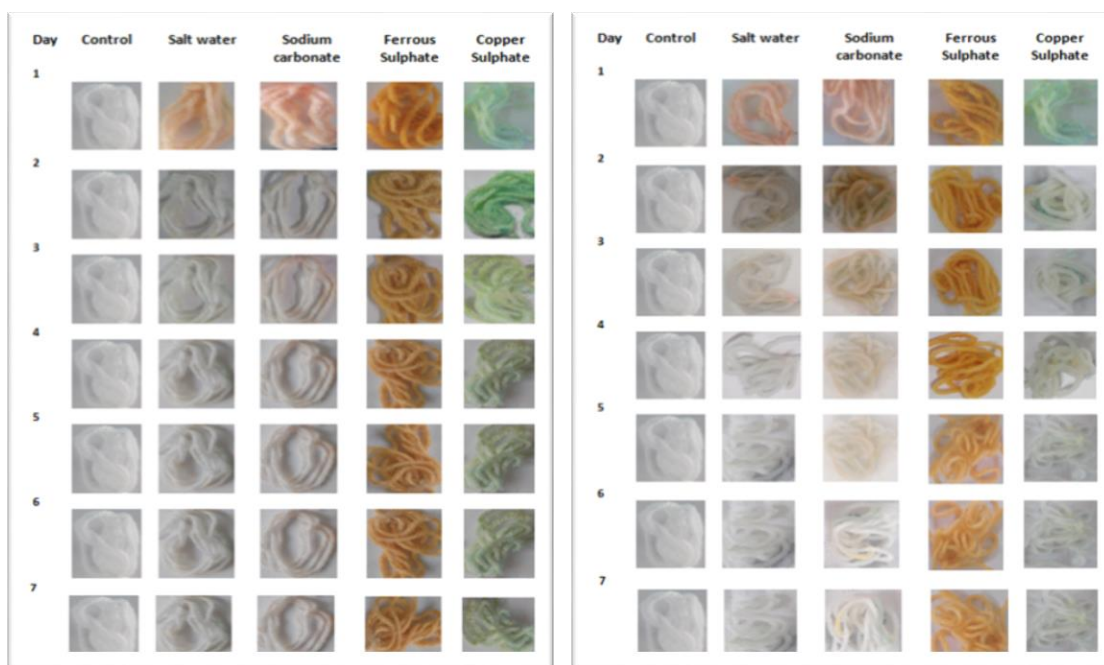


Figure 4: Results of dyed woollen fabric and exposure of light (left) & washing (right) consecutive 7 days

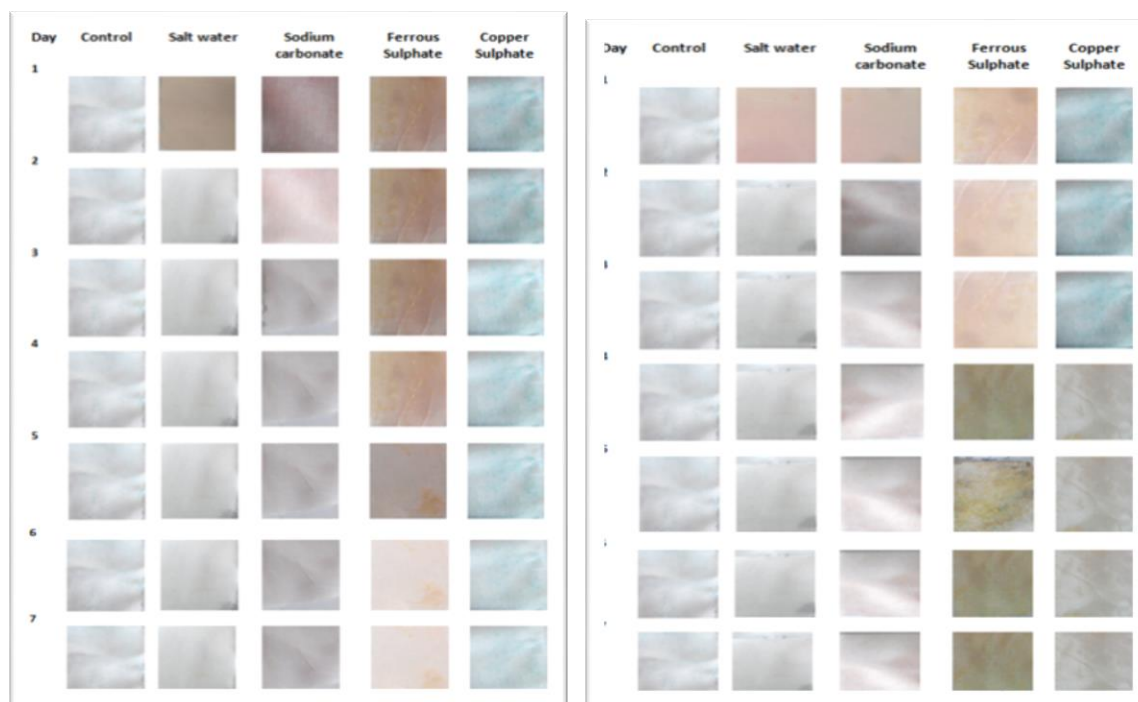


Figure 5: Results of dyed nylon fabric and exposure of light (left) & washing (right) for consecutive 7 days

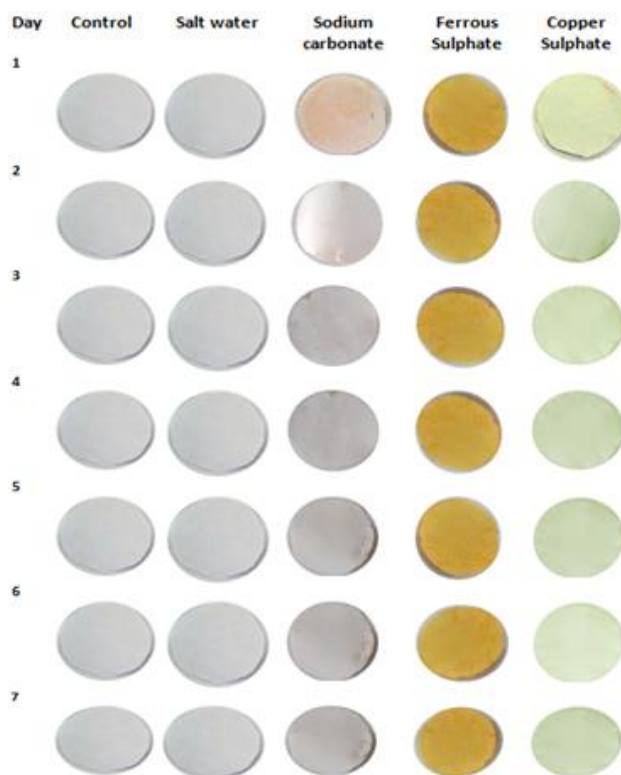


Figure 6: Results of dyed paper after exposure to light for 7 days



Figure 7: Microbial pigments as colourants in candle & bioplastics

6. CONFLICT OF INTEREST

The authors hereby declare that they do not have any conflict of interest for the research work communicated through this paper.

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Marine *Pseudomonas aeruginosa* MGPB31: A Potential Bioinoculum to Alleviate Salinity Stress in Spinach (*Spinacia oleracea*)

P. UCHGAONKAR, S. KUDALE, S. SINGH, D. DASGUPTA*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Salinity is one of the major environmental stresses that reduces plant growth. Inoculating plants with non-pathogenic bacteria can provide bioprotection against biotic stresses such as drought, salinity etc. In the current study *Pseudomonas aeruginosa* MGPB31 isolated from marine environment was assessed for its PGPR (plant growth promoting rhizobacterial) activity for Spinach (*Spinacia oleracea*). The application of this PGPR in pot culture studies exhibited a significant increase in morphological parameters viz root length, shoot length and total chlorophyll content as compared to the control. A parallel pot culture study conducted with 2% NaCl stress proved that the isolate also enhanced salinity tolerance in Spinach plants thereby helping the plant combat the salt concentration in the soil. The comparative analysis of both these studies indicates the marine isolate *Pseudomonas aeruginosa* MGPB31 has a PGPR potential for promoting plant growth under normal and salinity stress.

KEYWORDS: salinity, PGPR, Spinach, *Pseudomonas aeruginosa*

***Corresponding Author: Dr. Debjani Dasgupta**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: drdebjanid@dypatil.edu

1. INTRODUCTION

The indiscriminate use of fertilizers, particularly those containing high amounts of nitrogen and phosphorous has led to substantial pollution of air, water and soil. The excessive use of these chemicals exerts deleterious effect on soil bacteria thus affecting the fertility status of soil. The application of these fertilizers on a long term basis leads to reduction in pH of the soil and the productivity of the crop is affected [1]. This excessive use of fertilizers is also accompanied by expansion of irrigation to achieve self sufficiency in agriculture. To meet this ever increasing demand of irrigation, ground water has been exploited which has lead to an increase in the water table thereby increasing salinization [2]. It has been estimated that worldwide 20% of total cultivated and 33% of irrigated agricultural lands are affected by high salinity and by 2050, more than 50% of the arable land would be salinized [3]. Excess sodium in the soil competes with Ca^{+2} , K^{+} and other cations thereby reducing their availability to crops. Hence, soils with high levels of exchangeable sodium impact plant growth by dispersion of soil particles and imparting nutrient deficiency [4]. Towards a sustainable agriculture vision, crops need to be equipped with plant growth promoting rhizobacteria (PGPR) that can enhance plant health, growth and confer resistance against abiotic stresses [5,6,7].

Spinach is an important green vegetable that contains large quantities of bioactive compounds and nutrients that are not common to most other vegetables. It was identified as a moderately salt sensitive vegetable. Salt stress reduced spinach germination, root elongation and chlorophyll content and increased membrane permeability [8]. With regard to nutritional quality previous reports indicate that low fertilization could enhance nutritional values of vegetable crops. However, literature on the growth of spinach affected by abiotic stress is scarce [9]. The current study therefore aims to study the effect of salinity on the growth of spinach and a marine isolate of *Pseudomonas aeruginosa* as a possible bioinoculant that can help in alleviating salinity stress.

2. MATERIALS AND METHODS

2.1. Salt tolerance of the isolate used for PGPR activity

The isolate used to study PGPR activity was obtained from marine source and identified as *Pseudomonas aeruginosa* on the basis of morphological, biochemical and 16s rRNA sequencing. The culture was submitted to genbank as *Pseudomonas aeruginosa* strain MGPB31 under National Centre for Biotechnology Information (NCBI) accession number MF511820. This isolate was identified as a potent siderophore producer in modified M9 medium (MM9) and the amount of siderophore produced was quantified using the CAS shuttle assay [10]. As the isolate was obtained from marine source, apart from the siderophore producing ability of the isolate, the salt tolerance of the isolate was checked by streaking the isolate on Nutrient agar plate with different concentrations of NaCl (3% to 10%). All the plates were incubated at RT for 48 – 72 hours and observed for growth. The highest concentration of salt that exhibited growth was considered as salt tolerating ability of the isolate [11].

2.2. Soil used

The soil used in the study was Soilrite -Sativic Farms Special which is soil-less blend composed of imported Peat moss, Perlite, Vermiculite and Cocopeat. The soilrite mixture was autoclaved to make it sterile and also to moisten it so that it holds maximum water. This soil was used as it preserves moisture and is considered ideal for container gardening. It provides a sterile planting medium and retains much water than garden soils [12].

2.3. Collection of seeds

The spinach seeds variety *Harita* were bought from the local market of Navi Mumbai. The seeds were kept in a cool, dry and clean place in the laboratory for further use [13].

2.4. Seed germination test

The efficiency of germination of approximately 100 spinach seeds was performed in the PTC laboratory of the school. Seeds were surface sterilised with 1% sodium hypochlorite for ten minutes followed by successive washing with sterile distilled water to remove traces of sodium hypochlorite. The sterilization procedure is required to eliminate contamination by microorganisms during the germination process [14]. The surface sterilised seeds were inoculated by soaking them in a suspension of *Pseudomonas aeruginosa* MGPB31 of O.D 0.1 (10^8 cfu/ml)

containing 0.5% carboxymethylcellulose (CMC) as adhesive for 45 minutes [15]. The coated seeds were transferred and placed on sterile soilrite in plastic glass and incubated for 2 – 3 days. Seeds immersed in sterile distilled water served as control. After 3 days, seed germination was recorded in comparison to control. The seed germination rate was calculated using the formula [16]:

Germination rate (%) = (No. of germinated seeds / total no. of seeds) x 100

2.5. Effect of PGPR (*P. aeruginosa* MGPB31) on growth of Spinach:

The experimental set up consisted of 50gm sterile soilrite in plastic cups with 2.5gm of bactericised seeds. The control set consisted of seeds treated with sterile distilled water instead of bacterial suspension. All the experiments were set up in triplicates. The plants were watered everyday using 10ml sterile double distilled water. After 28 days of sowing, all plants were carefully uprooted and various vegetative parameters like root length, shoot length, wet and dry weight were studied. Estimation of chlorophyll was done using 80% acetone [17, 18]. From the nutritional point of view, the iron content of the plant was checked by Atomic absorption spectroscopy (AAS) [19].

2.6. Effect of 2% NaCl (Salinity stress) on Spinach:

To study the effect of salinity stress, for every 50gm of sterile Soilrite 2% NaCl was added at a final concentration. Bacterised seeds were sown as mentioned above in soilrite having 2% NaCl. A control was maintained where seeds treated with D/W were sown in Soilrite with 2% NaCl. The plants were treated and all the parameters were analysed as mentioned earlier. Apart from these parameters, the sodium content was determined in stressed as well as control samples [20].

3. RESULTS

3.1. Salt tolerance of the isolate

After 48 hours of incubation, growth was seen on Nutrient agar plates having NaCl concentration from 3% to 5%. However even after 72 h of incubation, no growth was seen on Nutrient agar plates having NaCl concentration more than 5%, thereby indicating the isolate to be tolerant upto 5% NaCl (Figure 1).

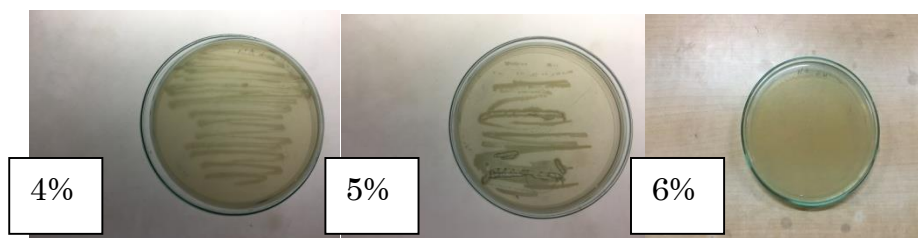


Figure 1: Growth of the isolate S31 on N/A with 4%, 5% and 6% NaCl respectively

3.2. Seed Germination test

A total of 100 seeds were studied for seed germination test. While the seeds treated with bacteria exhibited a higher germination rate (88%) as compared to the control (76%) indicating the applicability of the isolate as plant growth promoting bacteria.

3.3.Effect of PGPR on growth of Spinach

The effect of the isolate *P. aeruginosa* MGPB31 on the growth of Spinach was studied using pot culture studies. The results of the growth parameters studied have been depicted in Figure 2 and Table 1.



Figure 2: Effect of the isolate *P. aeruginosa* MGPB31 on growth of Spinach

	Root length (cm)	Shoot length (cm)	Wet weight (g)	Dry weight (g)	Chlorophyll content (mcg/ml)	Iron content (mg/g)
Test	3.5±0.5	7.4 ± 0.3	1.28 ± 0.4	0.25 ± 0.1	21.20± 0.5	3.97± 0.2
Control	1.6±0.2	6.3 ± 0.1	1.07 ± 0.2	0.15 ± 0.1	18.06± 0.3	3.11± 0.4

Table 1: Plant growth parameters observed for treated and control Spinach plants. Results presented are mean of triplicates ± S.D.

3.4.Effect of 2% NaCl (Salinity stress) on Spinach

Salinity in agriculture is a global problem specifically in arid and semi-arid regions owing to low rainfall and high evaporation. Salinity induces osmotic stress and ionic toxicity that leads to secondary oxidative stress in plants. Bacterial inoculants have recently been used for the amelioration of salt stress in crop plants [21]. Hence, in the current study, the potential of the inoculant *P. aeruginosa* MGPB31 was studied for its ability to ameliorate salt stress and promote plant growth under conditions of salinity. The presence of salinity reduced plant growth but the stress was relieved in plants inoculated with the isolate as compared to plants untreated with the isolate (Control) (Figure 3). The results of the effect of salinity on plant growth parameters are depicted in Table 2.

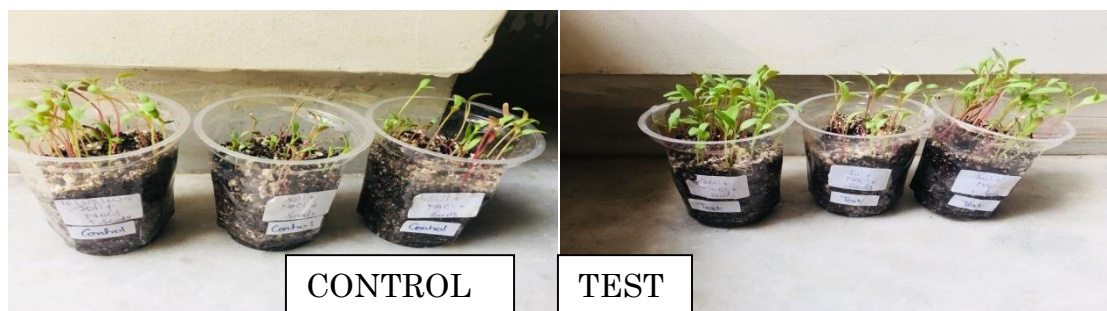


Figure 3: Effect of salinity stress (2%) on Spinach. Left: Control where plant growth is affected due to salinity Right: Test exhibiting increased plant growth as compared to control.

Parameters	Test	Control
Root length (cm)	3.4 ± 0.2	1.3 ± 0.1
Shoot length (cm)	7.3 ± 0.3	5.7 ± 0.2
Wet weight (g)	2.59 ± 0.5	1.76 ± 0.3
Dry weight (g)	0.325 ± 0.08	0.117 ± 0.06
Chlorophyll content (mcg/ml)	24.87 ± 0.05	21.12 ± 0.09
Iron content (mg/gm)	4.46 ± 0.15	1.26 ± 0.02
Sodium content (mg/gm)	0.012 ± 0.003	0.02 ± 0.001

Table 2: Effect of 2% NaCl on plant growth parameters of microbe treated and Control Spinach plants. Results presented are mean of triplicates $\pm$ S.D.

4. DISCUSSION

Siderophore producing plant growth promoting bacteria have shown to play a vital role in plant growth promotion and in increasing the iron nutrition of the plant. Hence, in the present study, the effect of marine bacterium *Pseudomonas aeruginosa* MGPB31 was assessed for its potential to increase iron content in plants as well as to alleviate salinity in plants growing in saline sodic areas. The marine bacterium *Pseudomonas aeruginosa* MGPB31 could tolerate a salt concentration as high as 5% however a concentration more than 5% inhibited the growth. Increase in salt concentration above 4% outside the cell membrane increases osmotic potential by creating hyper osmotic pressure which could be the reason for reduced growth of PGPR at concentrations higher than 4% [2]. The pot culture studies of *Spinacia oleracea* indicate that application of siderophore producing isolate *Pseudomonas aeruginosa* MGPB31 exhibited enhanced plant growth. A significant increase was seen in the root length, wet weight, dry weight and chlorophyll content of the treated plants. A 12% increase in the rate of germination, two fold increase in root length, 11 % increase in shoot length, and 3 % increase in chlorophyll content was observed in seeds inoculated with *P. aeruginosa* MGPB31 over control. Similar results have been reported by Sayyed *et al* [22] for the groundnut seeds inoculated with *Alcaligenes faecalis*. This is due to the siderophore production that confers competitive advantage to PGPR so that they can colonise roots and exclude other organisms from this ecological niche [23]. A significant increase (8%) was also observed in the iron content of treated plants as compared to those of the control plants. This is due to the solubilizing effect of siderophores on iron hydroxides that benefit plant iron acquisition. In this

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context, various microbial iron- siderophore complexes like iron-ferrioxime for oat, iron – pyoverdine for arabdiopsis, and iron – rhizoferrin for tomato, barley and corn have been reported by Jin *et al* [24]. Similar increase in iron content was observed in case of maize and canola plants when these plants were treated with siderophore producing bacteria [25].

Generally, plants follow two strategies to solubilise unavailable iron (ferric form) from the soil. In most of the plants referred as strategy I plants, a plasma membrane bound reductase is induced with enhanced secretion of protons that help in ferric (Fe^{+3}) uptake whereas in strategy II plants, there is an increase in the secretion of phytosiderophores that chelate ferric ion for entry in the cell [26]. Apart from increasing the iron content of plant, spraying of iron free siderophores on leaves could prevent pathogen attack, based on iron scavenging capacity of bacterial siderophores and the requirement of iron for other microorganisms [27]. The addition of 2% NaCl resulted in reduced growth of Spinach plants. Primary plants grown in the presence of NaCl showed upto 62% reduction of root length than of plants grown in absence of NaCl. A similar decrease (22%) was also observed in shoot length of control plants as compared to plants treated with the isolate. Reduction in root length causes a reduction in nutrient uptake by plants. This eventually leads to reduced plant growth and eventually reduced shoot length. The reduction in weight of plants under salt stress may be due to the inhibition or hydrolysis of reserved food and its translocation to growing shoot plants. Salinity stress diverts metabolic carbon to storage pools and as a result, less carbon is available for growth leading to reduced growth of plants [2].

Analysis of sodium content in plants reveal that the very less amount of sodium has been transferred in plants treated with isolate as compared to control plants. This could be one of the factors due to which plants treated with isolate exhibited better growth as compared to control plants. Similar results have also been reported by Tank and Saraf [2] where treatment with PGPR isolate lowers sodium uptake by plants thereby lowering the deleterious effects of sodium accumulation. The results obtained reveal that salinity caused an increase in sodium concentration but the amount accumulated in the treated plant was significantly low as compared to untreated plants.

5. CONCLUSION

Worldwide increase in population increases the growing demand of global food production and leads to environmental damage causing problems in agriculture yield. In the coastal areas, salinity also proves to be a major constraint which hampers agriculture productions in many areas. The use of salt affected soils for agriculture is indispensable to feed the over increasing population. Over the last 40 years, PGPR have been an effective tool towards sustainable agriculture [28]. The current study reports the PGPR activity of a marine bacterium *Pseudomonas aeruginosa* MGPB31 that exhibited siderophore production thus helping the plant in increasing its iron content. The salt tolerating ability of this isolate helped the plant to ameliorate salinity stress thereby minimizing the toxicity of stress conditions on plant growth. The results obtained suggest the use of this PGPR for enhancing plant growth in saline sodic areas to regenerate saline lands and maximize agricultural benefits.

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Production of Antioxidant by Marine *Pseudomonas stutzeri* KKB-1 and Assessing its Applications as an Anti-ageing Agent

N. SHIVALE, T. MARAR AND M. HARMALKAR*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Antioxidants combat the deleterious effects of oxidative stress by scavenging free radicals and thereby delaying the oxidation of cell macromolecules viz. DNA, lipids, proteins and carbohydrates. Marine microbial flora being constantly exposed to various stressed conditions viz exposure to UV radiation, discharge from industrial effluents etc, and these microorganisms could serve as potential antioxidant producers. In the current study, cell free supernatant (CFS) of marine isolate viz *Pseudomonas stutzeri* KKB-1 was assayed for its antioxidant potential by DPPH assay, FRAP assay and hydrogen peroxide radical scavenging assay. The percent DPPH radical scavenging activity of crude extracts of *Pseudomonas stutzeri* KKB-1 was 71.67% which was equivalent to DPPH activity of *Lactobacillus casei* and *Lactobacillus bulgaricus* viz. 73.81% and 76.99% respectively. The antioxidant activity as assayed by FRAP assay was found to be 7.08 ± 0.02 $\mu\text{g/ml}$ for crude extracts of *Pseudomonas stutzeri* KKB-1 and 9.46 ± 0.02 $\mu\text{g/ml}$ for both the standard *Lactobacilli* cultures. The hydrogen peroxide radical scavenging activity for the isolate and standard *Lactobacilli* cultures was in the range of 34% to 36%. The cytotoxic effect of the crude extracts of KKB-1 on fibroblast cell lines was assayed by MTT assay. No toxic effect was seen up to a concentration of 1mg/ml and the percent cell viability at this concentration was 75.37% which was equivalent to the percent viability shown by standard tannin. Moreover the extracts exhibited 42.22% anti-elastase activity, 33.59% anti-hyaluronidase and 14.35% anti-collagenase activity suggesting its application as an anti-ageing agent

KEYWORDS: *Pseudomonas stutzeri* KKB-1, antioxidant, *Lactobacilli*, anti-ageing

***Corresponding Author: Dr. Mugdha Harmalkar**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: mugdha.harmalkar@dyatil.edu

1. INTRODUCTION

Microorganisms are ubiquitous in nature and microbes existing in marine environments like estuaries, seawater and sediments, within or on the surfaces of macro-organisms and benthic structures etc are constantly exposed to extreme environmental conditions viz. freezing temperature (Antarctic waters), high temperatures (more than 100 °C) in deep sea hydrothermal vents, highly acidic conditions, discharge of industrial effluents etc. To combat damage caused by stressed conditions microorganisms produce antioxidants. Marine microorganisms isolated from sand sample, deep sea sediments, seaweeds and marine algae etc have been reported to produce antioxidants which help them to combat stress [1-4].

Antioxidants are known to play an important role in combating diseases such as cancer, diabetes and ageing [2]. Intracellular and extracellular oxidative stress initiated by reactive oxygen species (ROS) advance skin ageing, which is characterized by wrinkles and atypical pigmentation and is often related to UV exposure. The use of antioxidants is an effective approach to prevent symptoms related to photo-induced aging of the skin [4].

Soil samples near coastline areas are constantly exposed to stressful conditions such as temporary desiccation due to tidal patterns, exposure to UV irradiation, discharge of waste etc. These habitats which represent closely to marine and terrestrial environment have not been explored earlier [3]. In the present study we have evaluated the antioxidant potential of the cell free supernatants of marine isolate *Pseudomonas stutzeri* KKB-1 and compared its antioxidant activity with *Lactobacilli* cultures. We also studied the anti-ageing potential of the cell free supernatants of *Pseudomonas stutzeri* KKB-1 by biochemical assays.

2. MATERIALS AND METHODS

All the chemicals and reagents were procured from HiMedia Pvt. Ltd and Sigma Aldrich.

2.1. Bacterial cultures

Pseudomonas stutzeri KKB-1(KKB-1), a marine isolate [6], was maintained on nutrient agar medium. For experiment assays, loopful culture was inoculated in sea water complete (SWC) medium, incubated on shaker for 24 hours at room temperature. Standard *Lactobacilli* cultures viz. *Lactobacillus casei* and *Lactobacillus bulgaricus* were isolated and maintained in MRS medium.

2.2. Extraction of the antioxidants

Pseudomonas stutzeri KKB-1(KKB-1) culture was grown in sea water complete (SWC) medium for 24 hours at room temperature [7], and the cells were separated by centrifugation. The cell free supernatant was mixed with equal volume of ethyl acetate, mixed vigorously in separating funnel and left undisturbed 1 hour [8]. The organic solvent layer (upper layer) was collected and concentrated in a rotavapor at 60 °C. For comparing the antioxidant potential of KKB-1, standard antioxidant producing cultures viz. *Lactobacillus casei* and *Lactobacillus bulgaricus* were grown in MRS broth and cell free supernatants were subjected to ethyl acetate extraction method. The dried crude extract samples were reconstituted in 1 ml DMSO and used for antioxidant and anti-aging assays.

2.3. Antioxidant assays

2.3.1. DPPH assay

The DPPH assay of the reconstituted crude extract samples was performed as described by Blois, (1958) [9]. Ten micro litres of extract samples of *Pseudomonas* and 30 µl extract of standard *Lactic acid bacilli* were used for the assay. Ascorbic acid was used as standard [10]. The percent radical scavenging activity (RSA) was calculated as:

$$\text{RSA (\%)} = (A_0 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of control and
 A_1 is the absorbance of test sample.

2.3.2. FRAP assay

FRAP assay of the reconstituted crude extract samples of *Pseudomonas* and standard *Lactic acid bacilli* was performed as described by Chanda and Dave, (2009) [11] using ascorbic acid as standard.

2.3.3. Hydrogen peroxide radical scavenging assay

Hydrogen peroxide radical scavenging assay of the reconstituted crude extract samples was performed as described by Chanda and Dave, (2009) [11] using ascorbic acid as standard.

The percent radical scavenging activity (RSA) was calculated as:

$$\text{RSA (\%)} = (A_0 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of control and
 A_1 is the absorbance of test sample.

2.4.MTT Cytotoxicity assay

To evaluate the cytotoxicity of the reconstituted extracts, normal human dermal fibroblast cell line was used. 2500 cells were seeded per well in a 96 well plate and after 24 hours, cells were treated with different concentrations (0.1mg/ml, 0.5mg/ml, and 1.0 mg/ml) of reconstituted crude extract samples of *Pseudomonas*. After 24 hours of incubation, 10 μ l of MTT reagent (5mg/ml) was added to each well including the blank. The plates were kept at 37°C in dark. The medium was discarded carefully and the formazan crystals were dissolved in 100 μ l of SDS reagent. The plate was kept at RT for 30 min under shaker conditions and read at 570nm [12]. Tannin was used as standard. The IC₅₀ value was used for studying the anti-ageing effect by anti-elastase assay, anti-hyaluronidase assay and anti-collagenase assay.

2.5.Anti-ageing assays

Biochemical assays on skin aging related enzymes were done to check the inhibitory action of reconstituted crude extract samples of *Pseudomonas*.

2.5.1.Anti elastase activity

To study the anti-elastase activity of the extract, porcine pancreatic elastase was incubated with different concentration of reconstituted crude extract sample of *Pseudomonas*. The amount of enzyme left uninhibited was quantitatively detected by reacting it with substrate N-Succ-(Ala)-nitroanilide that gives p-nitroaniline as the final product. This was read spectrophotometrically at 410 nm [13]. Tannin was used as standard.

Calculation:

$$\% \text{ Anti-elastase activity} = (A_0 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of control and
 A_1 is the absorbance of test sample

2.5.2. Anti-Hyaluronidase activity

Hyaluronic acid is a high molecular weight polysaccharide made up of N-acetyl-D-glucosamine and D-glucuronic acid, found abundantly in connective tissue and holds water keeping the skin moist, lubricated and smooth. Hyaluronidase splits the hyaluronic acid leading to formation of N-acetyl glucosamine and decreases the moisture content. Hence, the anti-hyaluronidase activity of the extract was spectrophotometrically determined by measuring amount of N-acetyl glucosamine formed by action of bovine hyaluronidase on sodium hyaluronate in the presence of extract of *Pseudomonas* [14]. The reaction system was read spectrophotometrically at 585nm. Tannin was used as standard.

Calculation:

$$\% \text{ Anti-Hyaluronidase activity} = (A_0 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of control and

A_1 is the absorbance of test sample

2.5.3. Anti-Collagenase activity

Collagenase when incubated with bovine collagen for 5 hours at 37°C, pH 7.5 acts on collagen to release leucine which is measured colorimetrically using ninhydrin method. The extent of inhibition of collagenase activity by different concentration of extract of *Pseudomonas* was measured at 600nm [15].

Calculation:

$$\% \text{ Anti-collagenase activity} = (A_0 - A_1/A_0) \times 100$$

Where, A_0 is the absorbance of control and

A_1 is the absorbance of test sample

2.6. Statistical Analysis

All assays were performed in triplicate and the results were presented as the mean $\pm$ SE (standard error). The values of $P < 0.05$ were used to identify statistically significant differences.

3. RESULTS

The characteristics of the isolate under study, its biochemical tests and 16S rRNA identification has been discussed in our publication [6]. Accordingly the isolate was identified as *Pseudomonas stutzeri* KKB-1.

3.1. Antioxidant assays of the isolates and standard cultures

A comparison of the antioxidant potential of ethyl acetate extract of *Pseudomonas* was done with standard *Lactobacillus* cultures using antioxidant assays such as DPPH assay, FRAP assay and Hydrogen peroxide scavenging assay.

The percentage inhibition of DPPH by ethyl acetate extracts of *Pseudomonas* and standard *Lactobacillus* cultures was found to be about 71% to 77% (table 1). The percent radical scavenging activity expressed as ascorbic acid equivalence was found to be $211.5 \pm 0.01 \mu\text{g/ml}$, $263.33 \pm 0.002 \mu\text{g/ml}$ and $273.33 \pm 0.01 \mu\text{g/ml}$ for *Pseudomonas*, *Lactobacillus casei* and *Lactobacillus*

bulgaricus respectively. Thus the antioxidant potential of KKB-1 was comparable to standard *Lactobacilli* cultures. The statistical analysis by one way ANOVA indicates ($p < 0.05$), suggesting the data is significantly different.

The ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ complex thereby forming an intense blue Fe^{2+} -TPTZ complex with an absorption maximum at 593 nm is determined by Ferric reducing antioxidant power (FRAP) assay [16]. The antioxidant activity as determined by FRAP assay was 7 to 9 $\mu\text{g/ml}$ (table 1). The one way ANOVA test value $p < 0.05$ indicates the values to be significantly different.

Samples (Ethyl acetate extracts)	DPPH assay (% inhibition)	FRAP assay ($\mu\text{g/ml}$)	Hydrogen peroxide scavenging assay (% inhibition)
<i>Pseudomonas stutzeri</i> KKB-1	71.67 $\pm$ 0.007	7.08 $\pm$ 0.02	34.82 $\pm$ 0.02
<i>Lactobacillus casei</i>	73.81 $\pm$ 0.002	9.46 $\pm$ 0.02	34.78 $\pm$ 0.002
<i>Lactobacillus bulgaricus</i>	76.99 $\pm$ 0.01	9.46 $\pm$ 0.02	36.05 $\pm$ 0.003

Table1: The comparative analysis of the antioxidant potential of extracts of *Pseudomonas* and standard *Lactobacilli* cultures. The values are mean $\pm$ SD for triplicates. As per one way ANOVA $p < 0.05$

The hydrogen peroxide radical scavenging potential of reconstituted extracts was found to be 34% to 36% (table 1). The ascorbic acid equivalence was 230 $\pm$ 0.03 $\mu\text{g/ml}$ for *Pseudomonas* and 228.03 $\pm$ 0.002 $\mu\text{g/ml}$ and 236.35 $\pm$ 0.003 $\mu\text{g/ml}$ for *Lactobacillus casei* and *Lactobacillus bulgaricus* respectively. The statistical analysis by one way ANOVA ($p > 0.05$) indicating the values for *Pseudomonas* and standard *Lactobacilli* are not significantly different.

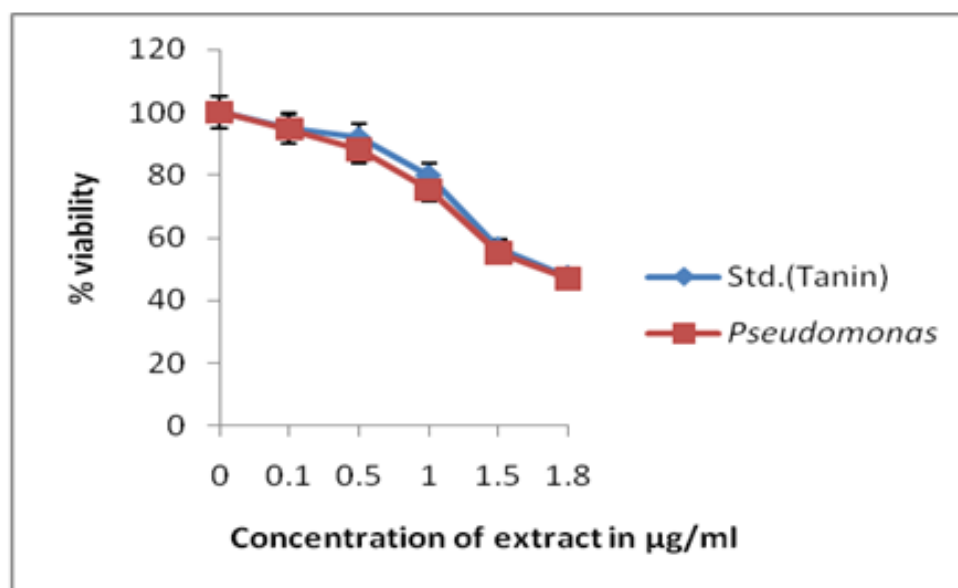
3.2.MTT cytotoxicity assay

To study the toxicity of the ethyl acetate extract of *Pseudomonas*, human dermal cell line was treated with 0.1mg/ml to 1.8 mg/ml of extract. No toxicity was observed up to a concentration of 1.00 mg/ml. The IC_{50} value of the ethyl acetate extract of *Pseudomonas* was 1.67 mg/ml which was equivalent to IC_{50} value of ethanolic extracts of standard tannin viz 1.60 mg/ml (Graph 1).

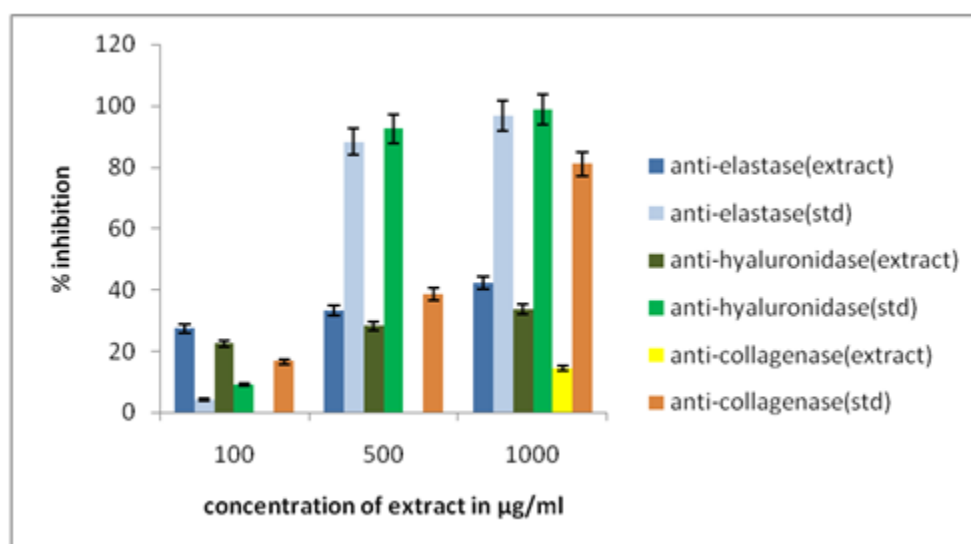
3.3.Anti-ageing assays

The anti-wrinkling effect of the ethyl acetate extract of *Pseudomonas* was studied by anti-elastase activity, anti-hyaluronidase activity and anti-collagenase activity. The percent inhibition of elastase enzyme increased with increasing concentration of extract. At a concentration of 100 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, the anti-elastase activity was 27.46 $\pm$ 2.56%, 33.28 $\pm$ 2.20% and 42.22 $\pm$ 0.99% respectively (Graph 2). The inhibition of the hyaluronidase enzyme was 22.49 $\pm$ 1.32%, 28.14 $\pm$ 1.70% and 33.59 $\pm$ 0.9% at the concentration of 100 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ respectively (Graph 2). The concentration dependent inhibition of elastase and hyaluronidase enzyme suggests the ethyl acetate extract of KKB-1 has anti-wrinkling and

moisturizing effect. No anti-collagenase activity was observed at 100 µg/ml and 500 µg/ml concentration of *Pseudomonas* extract however at 1000µg/ml concentration only marginal inhibition ($14.35 \pm 0.88\%$) of collagenase activity was observed. Thus the results indicate that the ethyl acetate extracts of *Pseudomonas* exhibit potential as an anti-wrinkling agent and moisturizing agent.



Graph 1: Percent viability of the human dermal fibroblast cells in presence of the ethyl acetate extract of *Pseudomonas*.



Graph 2: The percent inhibition of the anti-elastase, anti-hyaluronidase and anti-collagenase activity by the extracts *Pseudomonas stutzeri* KKB-I. Tannin was used as standard for all the assays.

4. DISCUSSION

In this study we report the antioxidant potential of ethyl acetate extracts of *Pseudomonas stutzeri* KKB-1 as assayed by DPPH assay, FRAP assay and Hydrogen peroxide scavenging assay.

The ethyl acetate extracts of *Pseudomonas stutzeri* KKB-1 exhibited 71.67% of DPPH radical scavenging activity and 7.08 ± 0.02 $\mu\text{g/ml}$ of antioxidant activity by FRAP assay. This antioxidant activity was comparable to antioxidant activity of ethyl acetate extracts of *Lactobacilli* cultures. The hydrogen peroxide radical scavenging potential of ethyl acetate extracts of KKB 1 was also found to be equivalent to standard *Lactobacilli* cultures, thus suggesting the marine isolate, *Pseudomonas stutzeri* KKB-1 as potential source of antioxidant. Shimel Wu, 2016 reported isolation of *Pseudomonas stutzeri* 273 from sediments of East China Sea and the exopolysaccharide produced by this organism showed antioxidant activity [17]. *Pseudomonas stutzeri* Nt-I isolated from drainage water of a selenium refinery plant in Hyogo, Japan is capable of reducing selenium [18, 19]. Thus *Pseudomonas stutzeri* species isolated from varied regions have shown different properties but our study is the first to report antioxidant production.

Antioxidant activities were also reported by other microorganisms isolated from marine environments. *Pseudomonas koreensis* isolated from Sargassum (brown algae) exhibited 47.3% DPPH activity and 5.9 ± 0.13 $\mu\text{g/ml}$ of FRAP activity at 1 mg of crude ethyl acetate extract [3]. The DPPH radical scavenging activity of extract of *Pseudomonas aeruginosa* MTCC 741 (200 μl) was shown to be 90% [20]. *Chromohalobacter israelensis* (GUVFCFM-3) a halophilic sponge biont was reported to produce extracellular antioxidant which scavenged 67.05% at a concentration of 1000 $\mu\text{g/ml}$ of DPPH free radicals and 67.21% of superoxide radicals [21]. The antimicrobial and antioxidant activity was reported in *Brevibacterium* species and *Corynebacterium* species isolated from sand sample collected from sea grass area [1] and *Bacillus simplex* XJ-25 isolated from sand biological crusts [2]. Thus our isolate viz. *Pseudomonas stutzeri* KKB-1 seems to have better antioxidant potential as compared to other reports.

Plant phytochemicals have been reported to have application as an anti-ageing agent by virtue of its ability to exhibit anti-elastase and anti-collagenase activity [22, 23]. Kim NY *et al*, 2015 reported the extracts of photosynthetic bacteria *Rhodobacter sphaeroides* and the pigment produced by this organism viz bacteriochlorophyll an exhibited high antioxidant activity attributed to the skin anti-aging effect [24]. In our study the ethyl acetate extracts of *Pseudomonas stutzeri* KKB-1 exhibited anti-elastase and anti-hyaluronidase activity and moderate anti-collagenase activity as compared to standard tannin thereby suggesting the potential role of extracts of *Pseudomonas stutzeri* KKB-1 as an anti-wrinkling agent and moisturizing agent.

5. CONCLUSION

The antioxidant activity of the ethyl acetate extracts of *Pseudomonas stutzeri* KKB-1 is comparable to the antioxidant activity of the extracts of standard *Lactobacilli* cultures. The IC_{50} of the ethyl acetate extract of *Pseudomonas* was observed to be 1.67 mg/ml, which is in accordance with the IC_{50} value of the extract of standard tannin viz. 1.6mg/ml. The extract has a moderate anti-wrinkling activity and hence can be considered as a potential candidate for cosmetic

formulations.

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7. CONFLICT OF INTEREST

The authors hereby declare that they do not have any conflict of interest for the research work communicated in this paper.

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Effect of Induced Short-Term Salt Stress at Seedling Stage on Antioxidant Enzymes among *Luffa* Varieties

M. PARAB, I. SYED, P. DE, S. SINGH*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Salinity, a key abiotic stress, impinges several vegetable crops and adversely delimits the plants morphological characteristics, crop yield, plant vigour, imbalance in cellular ions which imbibe ion toxicity. Osmotic stress thus created eventually embarks accelerated production of reactive oxygen species (ROS). As an adaptive mechanism; plants have developed antioxidant system; consisting of enzyme such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione reductase (GR). In present study, effect of induced short-term salt stress in two varieties of *Luffa acutangula* [a. Jaipuri Long (JL); b. Mumbai Local (ML)] was assessed. After a 48 hours of salt treatment (50 mM, 100 mM, 150 mM and 200 mM of NaCl), a cohort of antioxidant enzyme activities was evaluated to estimate the magnitude of salt tolerance. Results indicated difference according to the variety's ability to cope with oxidative stress caused by salinity. ML variety showed decrease in SOD activity with increasing salt concentration while in JL variety, enzyme activity impressively increased at 150 mM and 200 mM salt concentrations. CAT was scored to be more active in ML variety than in JL variety at higher salt concentrations. Both the varieties showed highest POD activity at 150 mM salt concentration. An increasing trend was seen in GR activity for JL variety while reciprocal for ML was recorded. Though peroxidase and CAT play an important role in ML for combating salt stress, it may not be much efficient as SOD activity decreased at higher salt concentrations. Hence, it may be proposed that JL variety could be more salt-tolerant than ML variety. Furthermore SOD is likely to be central in defence against ROS.

KEYWORDS: *Luffa acutangula*, Salinity, Osmotic imbalance, ROS, POD, SOD, CAT, GR.

***Corresponding Author: Dr. Sunita Singh**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: dr.sunitads@gmail.com

1. INTRODUCTION

Salinity stress, unconstructively effects plant's morphological characteristics, crop yield and plant dynamism causing a cellular imbalance as well as ion toxicity [1, 2]. It also leads to stomatal closure, thereby reducing CO₂ availability in leaves and rate of carbon fixation [3]. Induction of stress conditions lead to production of higher amounts of ROS which require more potent enzymatic and non-enzymatic antioxidant system (AOS) to ameliorate its deleterious effects [4]. Higher levels of ROS lead to accumulation of hydrogen peroxide, inhibition of photosynthetic rate, damage to plasma membrane permeability, reduced chlorophyll content, etc., eventually causing

cell death. Enzymatic anti-oxidant system comprises of enzymes such as catalase, superoxide dismutase, peroxidase, peroxiredoxin, glutathione reductase and glutathione sulphy-transferase while non-enzymatic AOS system consists of low molecular weight antioxidants like ascorbic acid, glutathione (GSH), tocopherol and Carotenoid [5, 6]. Catalase (CAT), peroxidase (POD), glutathione reductase (GR) and superoxide dismutase (SOD) are among the key enzymatic antioxidants. SOD catalyzes the dismutation of superoxide ($O_2^{\bullet-}$) to H_2O_2 and O_2 . CAT and POD catalyze conversion of H_2O_2 to H_2O and O_2 whereas GR catalyzes the NADPH dependent reaction of disulphide bond of Glutathione disulphide (GSSG). GR is important in maintaining the GSH pool [7].

Luffa acutangula (L) Roxb, also called as ridge gourd, angled gourd, is a potential herbs found in Asia. It is common fruit vegetable cultivated in India, China, Southeast Asia, Egypt, Japan and other parts of Africa [8]. The multifarious medicinal and pharmacological chattel includes antidiabetic, antioxidant, hepato-protective and anti-cancer activity [9]. *L. acutangula* is also a very good source of the secondary metabolites like flavonoids, anthraquinones, terpenoids, tannins, and saponins [10]. It is a treasure chest of varied nutrients and anti-oxidants, hence also called the nutrition powerhouse. The raw fruit is consumed as an edible vegetable while the ripe fruit acts as a natural cleansing sponge as the fruit pulp converts into a dry mesh like structure on ripening. The crop being grown across the country has to adapt to vivid variations of edaphic and climatic conditions such as dry climate, high acidity, low water availability, etc., which eventually lead to biochemical changes in enzyme activity, isozyme pattern, metabolite pool, etc [11]. Making an allowance for the medicinal and pharmacological applications of *L. acutangula*, the present study endeavours to evaluate the activity of four key enzymes of AOS; namely CAT, SOD, POD and GR, in response to salt stress (NaCl) in two varieties of *L. acutangula* i.e. Jaipuri long (JL) and Mumbai local (ML) cultivated widely in India at seedling stage. In our previous studies [11], we have gauged these enzymes post four weeks of salt treatment in same varieties of *Luffa*. The effort allowed us to understand the vital AOS enzymes involved in ameliorating salt stress in these two varieties of *Luffa* under different salt stressed condition.

2. MATERIALS AND METHODS

2.1.Plant Material

The seeds of *Luffa acutangula* varieties a) Mumbai Local variety (ML) and b) Jaipuri Long variety (JL); were obtained from Namdeo Umaji Agritech (India) Pvt. Ltd, Mumbai. The seeds were surface sterilized, water imbibed (48 hrs) and treated with varying concentration of NaCl i.e. 50 mM to 200 mM. A set of control seeds was also taken which were not subjected to salt treatment. Each set comprised of six seeds.

2.2.Estimation of Antioxidant enzymes

For estimation of CAT activity, the crude extract comprised of 0.1g of seeds (salt treated & control) homogenised in 50mM potassium phosphate buffer (pH 7.0). The enzyme activity was estimated as described by Patel *et.al.* [11] Crude extract of SOD was synthesised by grinding 0.1g of seeds (salt treated & control) in 0.5 M Tris-HCl, 3 mM $MgCl_2$ and 1 mM EDTA (pH 4.5) buffer and

enzyme activity was estimated by method described by Kakkar *et. al.* [12] For POD, the enzyme was extracted from 0.1g of seeds (salt treated & control) in 0.1 M tris-HCl solution (pH 7.5) and the activity was estimated by method described by Noreen *et.al.* [13]. Glutathione reductase was extracted in 5% TCA, The enzyme activity of GR was calculated adopting method described by David and Richard [14]. The total protein content in crude extracts was estimated employing methods described by [11].

2.3.Statistical Evaluation

All the parameters studied were analyzed statistically using Sigma Stat Statistical Package (Version 14.0). One-way ANOVA with $P < 0.01$ was considered significant; Posthoc-Fischer analysis was done to test the concentration and varieties of statistical significance.

3. RESULT AND DISCUSSION

Salinity, a key abiotic stress, affects plants growth and development by reducing the water uptake from the soil and an increased absorption of ions especially Na^+ and Cl^- which meddle up with the various metabolic processes of the plant [15]. Osmotic stress and salinity were shown to enhance the production of ROS and cause ROS-associated injury [16]. These can seriously disrupt customary metabolism through oxidative damage to lipids, protein and nucleic acids. This leads to change in selective permeability of bio-membranes and thereby membrane leakage and change in the activity of enzymes bound to membrane occurs [17]. Sustenance of plants under salinity stress involves a consortium of cellular metabolites, enzymic as well as non-enzymic, that may act by controlling water loss through stomata, ion sequestration, metabolic adjustment, osmotic adjustment and anti-oxidative defence [15]. Consequently understanding the biochemical basis of salt-stress tolerance mechanisms is essential for breeding and genetic engineering of salt tolerance in plants. In spite of exhaustive research over the past several decades, which has improved our understanding of plants' response to salinity, there have been few studies on the mechanisms of the adaptation of vegetable crops to salinity. In present study two varieties of *L. acutangula* (ML, JL) were investigated for the response of CAT, SOD, POD and GR under induced salinity at seedling stage.

The challenge by NaCl resulted in imbalance of antioxidant enzyme activities at seedling stage and each enzyme had different response to NaCl treatment. Both the varieties demonstrated a reduced rate of germination with the increasing salt concentration (0- 200 mM). It was observed that ML had negligible or no germination at 150 mM and 200 mM salt concentrations while, JL exhibited germination in a diminishing trend at all salt concentration (fig. 1). CAT activity was scored to be higher in ML than JL among all the salt concentrations (50 mM, 100 mM, 150 mM and 200 mM). A slight increase (at 50 mM NaCl), followed by a decrease in CAT activity was recorded in ML variety. The skewness in concentrations of CAT can be attributed to the fact that plants possess multiple CATs encoded by specific genes, which respond differentially to salinity stresses [18]. However in JL variety, the concentration of CAT was observed to decrease with induced salt stress, which was much lower than the control plant (fig. 2). The results observed are in concordance with previous studies of our group Patel *et.al.* [11], wherein CAT activity

demonstrated a significant decrease post four weeks of salt treatment. Diminution in catalase activity can be attributed to fact that, CAT is known to play a major role in the photorespiration, where it prevents accumulation of ROS in the green tissues and thus delimit photo-inhibition of light-dependant Photosystem II in stress conditions [6]. Similar observation was reported by Shim *et al.* [19], in rice cultivars and have also been discussed by several other researchers in different other plant cultivars *viz.* Maize, Sunflower [13]. Further, the activity of this enzyme was higher in salt treated sample than in the control samples. Thus the present study showed that the ML variety appears to be more sensitive to stress and higher activity of CAT was exhibited in an attempt to combat salt stress conditions.

Conversely, the activity of POD was scored to be moderately increased in JL variety at 50 mM and 150 mM NaCl, while in ML variety an exclusive upshot in POD concentration was recorded at 150mM of NaCl as compared to control plant (fig.3). The differences in the POD enzyme activities of seedling may, at least in part explain the greater tolerance of JL variety to NaCl as compared to ML variety. The observations are in concord with those of Patel *et.al.* [11], who had reported a decrease in POD activity among 4 week old *Luffa* plants exposed to salinity stress. A remarkable increase of POD activity at 50mM of NaCl has also been reported by Meratan *et.al.* [20] amongst *Acanthophyllum sordidum*. Furthermore POD is actively involved in catalysing oxidation of phenolic and endiolic compounds with substrates such as hydrogen peroxide (H_2O_2) in cytosol, vacuole and cell wall to protect the cells against destructive influences of H_2O_2 under different abiotic stresses [21]. Thus, variability in levels of POD activity may be a manifestation of oxidative reactions corresponding to higher H_2O_2 level in plant cells with increased salinity.

The activity of SOD at seedling stage in JL variety was observed to decrease initially at lower salt concentration, while an increase was recorded at 150 and 200 mM of NaCl, while ML variety exhibited a steep decrease in SOD activity with increasing salt concentration (Fig. 4). On contrary, lab studies in the plants of same varieties have shown to have escalated SOD activity at lower salt concentration, depleted SOD activity at higher salt concentration post four weeks of salt treatment [11].

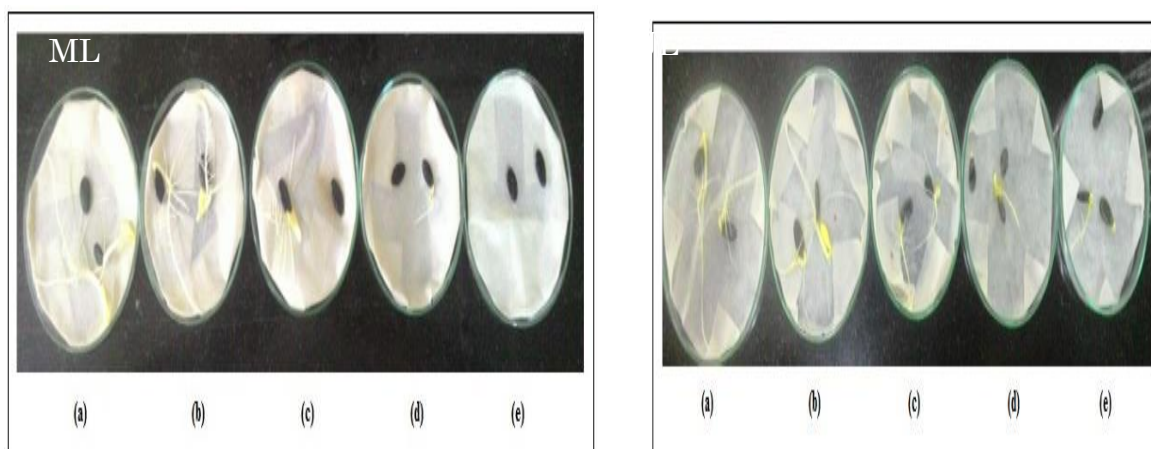


Figure 1: Seed germination in *Luffa* varieties at varying salt concentrations; (a) control, (b) 50 mM, (c) 100 mM, (d) 150 mM, (e) 200 mM of NaCl.

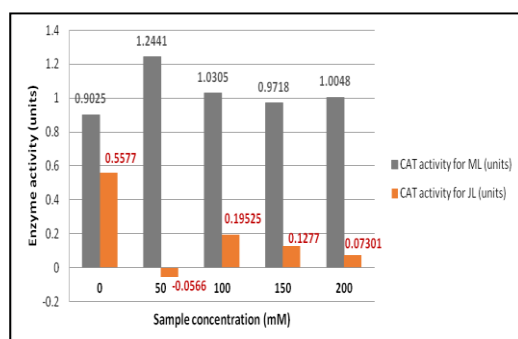


Figure 2: CAT activities of ML and JL var.

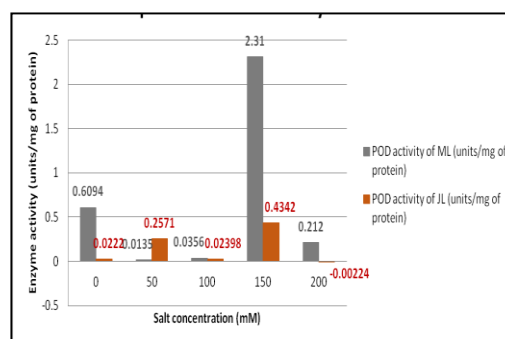


Figure 3: POD activities of ML and JL var.

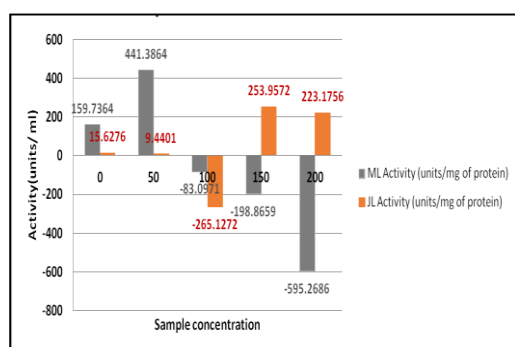


Figure 4: SOD activities of ML and JL var.

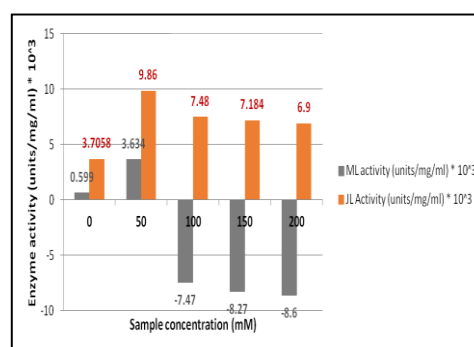


Figure 5: GR activities of ML and JL var.

It has been reported that salt stress induces oxidative damage to plant tissues [22]. The oxidative stress is considered to be due to increased production of free radicals like $O^{\cdot-}_2$, $OH^{\cdot}$ and non-radicals like H_2O_2 and $^1O_2$. Superoxide dismutase catalyses the dismutation of superoxide radicals to H_2O_2 and O_2 , and constitutes the most important enzyme in cellular defence because its activation directly modulates the amounts of O_2 – and H_2O_2 [23]. Thus constitutive and/or induced SOD expression at higher concentrations of NaCl may help plant to combat highly active oxygen species. On the contrary to JL, in ML variety, an upshot of SOD activity was recorded at 50 mM of NaCl, while at higher salt concentration an inverse correlation was exhibited. A decrease or no change in SOD activity has also been reported by Cavalcanti *et al.* [21], in cowpea leaves. Also a significant decrease in sensitive cultivars of rice was observed by Dionisio-Sese & Tobita, 1998. Furthermore diminished function of SOD may be due to a combination of the influence of subcellular location of the enzyme along with down regulation of genomic sequences and/ or reflective of competitive inhibition among isoforms of SOD [11].

Salinity stress initially increased GR activity at 50 mM of NaCl, while with increase in NaCl concentration, the GR activity was recorded to deplete. However the GR activity was observed to be elevated than that in the control plant of JL variety of *luffa*. Similarly in ML variety an initial increase in GR activity at 50 mM of NaCl was observed (fig. 5). With further increase in salt concentration the GR activity appeared to be downregulated in ML variety. On contrary to this in

our previous studies [11] GR activity was observed to increase indirect proportion to increased salt concentration. The ratio between the reduced and oxidized forms of glutathione (GSH/GSSG) is critical for activation of numerous defence mechanisms. The GSH/GSSG redox states thus appear to ephemerally shift towards a slightly more oxidized form of GR, in both varieties of *Luffa* in current study. Previous studies by Foyer *et.al.* [24], have reported that an upshot in GR activity boosts the reduction state of the glutathione pool and thus increase ascorbate regeneration. Ascorbate is an important non-enzymatic antioxidant, which was known to scavenge O_2^- , H_2O_2 and $\bullet OH$ [25]. Furthermore elevated levels of GR activity have shown to amplify the ratio of $NADP^+ / NADPH$, in that way ensuring the availability of $NADP^+$ as an acceptor of electrons. Hence the formation of new oxygen radicals shall get minimized and H_2O_2 conversion to H_2O via ascorbate-glutathione pathway by GR along with ascorbate peroxidase (APX), monodehydratescorbate reductase and dehydratescorbate reductase may get maximized [26]. In our study it has been thus observed that both the varieties of *luffa* (ML & JL) plants have developed their own unique mechanisms of AOS to combat negative effects of ROS. The flux in concentrations of AOS defence representatives i.e. CAT, POD, SOD and GR at seedling stage of both varieties of *Luffa* studied herein were observed to be statistically significant ($p < 0.01$) and were thus can be proposed as a consequence of induced salinity stress. Though the aforementioned enzymes have been shown to play key roles in the defence system, the exact adaptive mechanism has not yet been discovered. Several researchers have been trying to decode the blueprint of this mechanism which provokes deep diving into the study of enzyme pathway.

4. CONCLUSION

The present results indicate that salt stress modulates antioxidative enzymic responses of *Luffa acutangula*, in both ML as well as JL variety. The saline (NaCl) stress can impede seed germination and seedling growth among *luffa* varieties. Different concentrations of NaCl exhibited a skewed response (increase / decrease/ rebound) of enzyme activities of CAT, POD, GR, SOD, may be due to differential gene response at different plant tissues / organelles and/ or isoform competitive inhibition amongst these enzymes. Thus further studies on gene expression levels of each enzyme to confirm their responses under salinity are thus recommended.

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6. CONFLICT OF INTEREST

All the authors hereby declare that there is no conflict of interest.

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Poly-Hydroxy Butyrate Production using Agro Waste and Synthesis of Nano Biopolymers and its Application in Drug Delivery System

A. KHANDAVALLI, R.POL*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: PHB is biodegradable polyester, a substitute for plastics and has widespread applications. A study was conducted to obtain maximum PHB production using agricultural waste namely; orange peels, banana peels, bagasse. The production media was optimized with varying the physiochemical parameters like pH of the media and the incubation period and the concentrations of carbon sources, sodium chloride concentration. PHB producers isolated from marine samples and identified as *Bacillus pumilus* and *Enterobacter spp* were used for the study. The biodegradable polymer has been used in biomedicines in drug delivery system because of their biocompatibility and biodegradability. The other aspect of our study included the production of PHB nano particles using precipitation method by solvent displacement technique. The main objective of this research was to prepare nanoparticles of polymer loaded with the drug ofloxacin and to identify the drug release pattern. Ofloxacin has poor water solubility and low bioavailability and therefore was used for the in vitro drug release study. The optimization study revealed maximum PHB production using banana peels with 2% NaCl at pH 7 for an incubation period of 24 hours. The structural and morphological studies of PHB (drug unloaded) and PHB-Ofloxacin (drug loaded) nanoparticles were done using field emission gun scanning electron microscope (FEG-SEM). The drug loaded polymer existed a sustained release drug delivery form

KEYWORDS: Polyhydroxy butyrate (PHB), biodegradable, nanoparticles, polymer, in vitro drug release

***Corresponding Author: Dr. Reshma Pol**

School of Biotechnology and Bioinformatics, Dr. D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: reshma.kamble@dypatil.edu

Tel No: 022-394860

1. INTRODUCTION

Polyhydroxybutyrate is a biodegradable biopolymer produced from microorganisms. It is an alternative for petrochemical plastics and helps deal with the hazards of waste disposal problems. PHB is linear polyester accumulated in intracellular granules by a wide variety of gram-positive and gram-negative organisms under conditions of stress and nutrient limitation [1]. The marine environment is a promising resource for the discovery of PHB producers. In microbes producing PHB, the synthesis takes place if a suitable carbon substrate is available in excess and cellular growth is limited by other nutrients like nitrogen or phosphorus [2]. The major problem posed for PHB production from microbes is the high production cost. The carbon source itself attributed to

50% of the total costs, thus it is desirable to find a cheap alternative carbon feedstocks in order to improve sustainability of PHA production while obtaining commercial viability through fermentation. As such, inexpensive carbon sources such as agro-industrial by-products and agricultural residues have been reported as a potential alternative substrates [3] Different agricultural crops generate a lot of agro-wastes, e.g. Bagasse from sugarcane, wheat bran and rice bran, vegetable oil cakes, peels of potato and different fruits like orange, banana, etc. [4]

Nanotechnology-based delivery systems are known as a powerful method for *in vitro* and *in vivo* drug transportation. Polymeric nanoparticles are solid colloidal particles that range in size from 1 to 1000 nm. Application of biodegradable polymeric materials for preparation of nanoparticles allows controlled release at the targeted site. Poly-hydroxybutyrate has wide variety of applications as scaffold in regenerative medicine, packaging material in food industry, or nano-carrier for drug delivery. PHB is biodegradable under environmental conditions. Moreover, PHB does not induce inflammatory reaction *in vivo* and is fully biocompatible. The main objective of this research is to utilise *Bacillus pumilus* and *Enterobacter spp* for production of PHB using agro waste, optimization of physiochemical culture conditions to obtain maximum production. The synthesis of PHB nanoparticles and their use in the formation of nanobiopolymers which can be used for drug delivery system which was studied using *in vitro* drug release study.

2. MATERIAL AND METHODS

2.1. Microbial culture

The microbial cultures for this study were obtained from School of Biotechnology and Bioinformatics, D.Y Patil Deemed to be University. These organisms were isolated from the marine water samples and were identified as *Bacillus pumilus* and *Enterobacter spp*. These microbes were confirmed by gram staining and visualization of PHB granules by using phase contrast microscope. The chemicals were obtained from S.D. Fine chemicals Ltd., Mumbai were used for the production of PHB and synthesis of PHB nanoparticles.

2.2. Extraction and Quantification for PHB production

Saline suspension of bacterial inoculums *Bacillus pumilus* and *Enterobacter spp* were grown in carbon rich medium. The medium was prepared using (NH₄)₂SO₄, 5g/L; KH₂PO₄ 3.32g/L, MgSO₄·7H₂O, 3 g/L; Citric Acid, 0.42 g/L; trace element solution, 0.1 ml/L containing FeSO₄·7H₂O 0.25g/L; ZnSO₄·7H₂O, 0.0157g/L; CuSO₄·5H₂O, 0.005 g/L; MnSO₄·5H₂O, 0.02 g/L; CaCl₂·2H₂O, 0.0023 g/L; Na₂B₄O₇·10H₂O, 0.0023 g/L; (NH₄)₆Mo₇O₂₄, 0.001 g/L; pH 7.2 was autoclaved.

Using sodium hypochlorite method, fermentative samples were centrifuged at 10,000 rpm for 10 minutes and then washed with acetone and ethanol. The pellet was suspended in 4% sodium hypochlorite and was incubated at room temperature for 30 minutes. The suspension was again centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with acetone and ethanol. 10ml chloroform was added to the pellet by placing it in hot water bath at 60 °C to separate PHB from liquid broth. Chloroform was evaporated to obtain PHB crystals.

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracted PHB (g/L)} \times 100\%}{\text{CW (g/L)}}$$

The extracted PHB from isolates was dissolved in 10ml concentrated H₂SO₄ and was heated in water bath at 60°C for 1 hour to complete the conversion of PHB crystals into crotonic acid. The samples were cooled and vortexed. The amount of PHB was determined spectrophotometrically at 234nm against H₂SO₄ as blank using 100µg/ml of crotonic acid as standard. [5]

2.3. Microscopic visualization of PHB granules

Microbial cultures were cultured in the nutrient agar medium. The plate was incubated at 37°C. 0.42 gm% of Sudan black B prepared in ethanol was poured onto the culture plate and was left for 15min. Loop full of the culture was observed under phase contrast microscope.

2.4. PHB production using selected agro-wastes

One percent (v/v) aqueous extracts of dried powders of orange peels, banana peels, bagasse was used as a carbon source instead of citric acid in production medium. Growth of the organisms *Bacillus pumilus* and *Enterobacter spp* for the production of PHB was checked for 24h, 48h, and 72h interval of time. The PHB formed within cells was extracted using sodium hypochlorite method. Broth was centrifuged at 10,000 rpm for 15 minutes and the biomass was collected and weighed.

2.5. Effect of Initial pH and NaCl concentration

Effect of varying pH of media (5,6,7,8,9,10) and effect of different NaCl concentrations (1%,2%,3%,4%,5%) was observed for maximum PHB production by both the microbial cultures. After 24h of incubation, PHB was quantified spectrophotometrically using crotonic acid assay method.

2.6. Synthesis of PHB nanoparticles

PHB nanoparticles were synthesized by nanoprecipitation method by solvent displacement technique with slight modifications [6]. 10mg of extracted PHB was added with constant magnetic stirring in 40ml distilled with 1% Tween 20. It was centrifuged at 15,000 rpm for 1h. The supernatant was discarded and the pellet was air dried in incubator at 37°C for 3h. White PHB nanoparticles were characterized using SEM (scanning electron microscope).

2.6.1. Synthesis of In Situ (Drug Loaded) PHB-Ofloxacin Nanoparticles

PHB - Ofloxacin nanoparticles were prepared by solvent diffusion method. 10ml of Tween 20 was added to the 20ml of ethanol. To this, 10ml oil and 10ml of 5% drug was added. This was solution A. Solution B was made of 1% PHB nanoparticles dissolved in distilled water. Solution A and solution B were mixed for 2h using separating funnel. PHB-ofloxacin nanoparticles were obtained in the aqueous layer. The nanoparticles were air dried at 37°C for 3h and nanoparticles were characterized using SEM (scanning electron microscope)

2.7. Drug Release Study

The drug release studies were carried out in 0.1 M phosphate buffer solution using dialysis membrane. Initially, the dialysis bag was treated with phosphate buffer for 15 minutes. 50mg of PHB-ofloxacin nanoparticles were added to dialysis bag followed by addition of 1ml phosphate buffer solution and the set up was immersed in 50ml phosphate buffer solution (pH 7.4). The entire setup was kept under magnetic stirring at room temperature. After every half an hour, 1ml aliquot was withdrawn and the absorbance was measured at 293nm. Aliquots were withdrawn from time intervals to ensure the release of drug from the dialysis bag. [6,7]

3. RESULTS AND DISCUSSION

3.1. Extraction and Quantification for PHB production

The PHB accumulation using citrate as the sole source of carbon in the production medium for both the bacterial cultures was found to be 25.23% for *Bacillus pumilus* and 18.50% for *Enterobacter spp*.

SAMPLE NO	CDW (g/L)	PHB (g/L)	PHB (%)
<i>Bacillus pumilus</i>	0.004	0.013	25.23
<i>Enterobacter spp</i>	0.005	0.011	18.50

Table 1: PHB production and CDW (cell dry weight) obtained for *Bacillus subtilis* and *Enterobacter spp*

The quantification method involves the breakdown of the PHB polymers into its monomers. These monomers are converted to a compound that was measured using crotonic acid as the standard on a calibration curve. It was found to be 0.180 for *B. pumilus* and 0.105 for *Enterobacter spp*.

3.2. Microscopic analysis of PHB granules

The microscopic analysis was carried out using phase contrast microscopy that indicated the presence of PHB granules inside the bacterial cells.

3.3. Production of PHB using agro-waste

The PHB production using agro-wastes like dried powder of orange peels, banana peels and bagasse was used as a carbon source instead of citric acid in production medium. The residual biomass and the PHB concentration for *Bacillus pumilus* and *Enterobacter spp* was measured for three consecutive days of 24h intervals for three days.

Bacillus pumilus showed increased production of PHB for 24h (24.13%), 48h (24.68%), and for 72h (24.16%) using banana peel extract. Bagasse showed increased production of PHB from 24 h to 72 h (20.57% to 24.41%) For banana peel extract, *Enterobacter spp* showed decreased PHB production from 24h to 72h (24.96% to 20.9%) and for bagasse it was found to be constant from

24h to 72h (20.2% to 20.5%)



Figure 1. Microscopic visualization of PHB granules of *Bacillus pumilus* phase contrast microscope

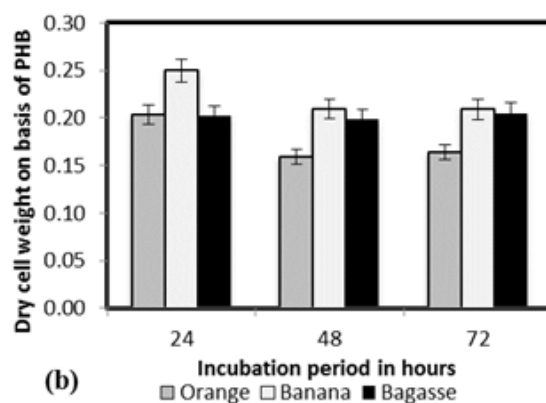
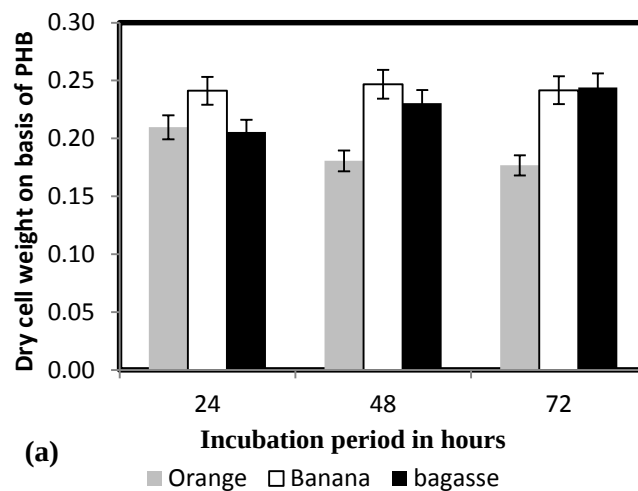


Figure 2: (A) Effect of PHB production using agro waste for *Bacillus pumilus*.(B) Effect of PHB production using agro waste for *Enterobacter Spp*

Figure 2A shows that for *B. pumilis*, banana peel extract showed increased production after 24h, 48h and 72h in comparison with other agro wastes used. Similar results were obtained for *Enterobacter spp* after 24h, 48h and 72h using banana peel extract which is indicated in Figure 2B.

3.4. Effect of Initial pH on the production of PHB on *Bacillus pumilus* and *Enterobacter spp*

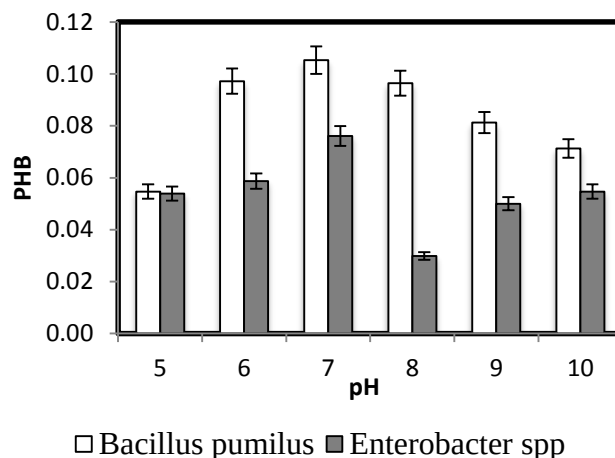


Figure 3. Effect of initial pH on PHB production of *Bacillus pumilus* and *Enterobacter spp*.

Fig 3 indicates that highest PHB production was at pH 7 for both the cultures of 10.53% for *Bacillus pumilus* and 7.61% for *Enterobacter spp*.

3.5. Effect of NaCl concentration on the production of PHB on *Bacillus pumilus* and *Enterobacter spp*

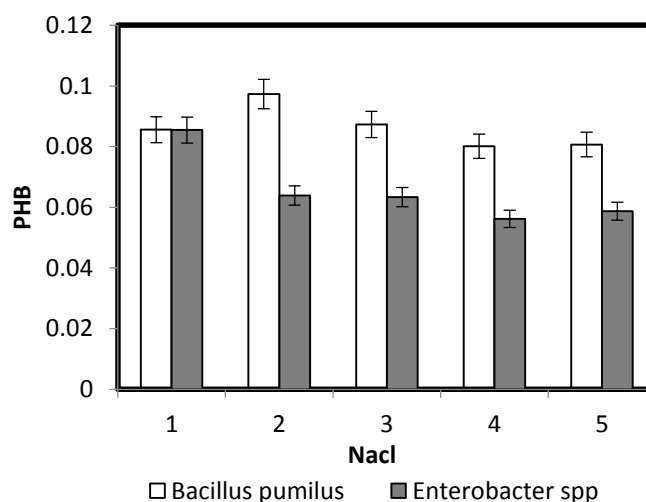


Figure 4. Effect of NaCl on PHB production of *Bacillus pumilus* and *Enterobacter spp*.

Fig 4 shows PHB accumulation where the highest production was observed at 2% NaCl concentration for *Bacillus pumilus* 9.73%, and at 1% for *Enterobacter spp*. of 8.55%

3.6.Synthesis of PHB nanoparticles

The PHB nanoparticles were synthesized using precipitation method by solvent displacement technique. The surface and morphological characterization of Polyhydroxy butyrate nanoparticles was carried out using scanning electron microscopy. Fig. 5 indicates the SEM images of PHB, prepared by nanoprecipitation method. The size of the particles was found to be in the range of 30-40nm for drug unloaded PHB nanoparticles. Fig. 6 indicates the particle size to be in the range of 17-35nm for drug loaded nanoparticles.

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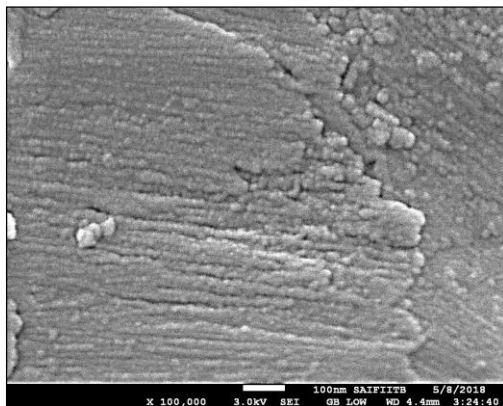


Figure 5: SEM image obtained for PHB drug unloaded nanoparticles at 100,000 X magnification

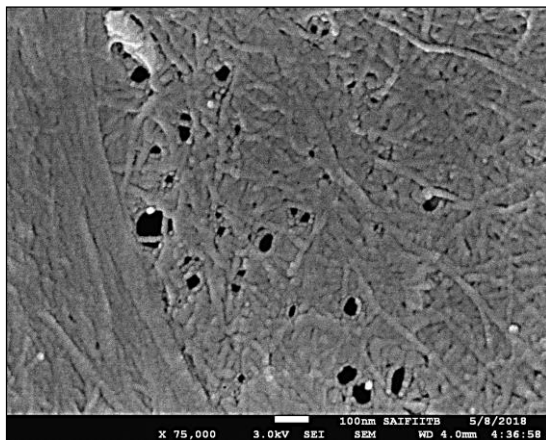


Figure 6: SEM image obtained for PHB-ofloxacin drug loaded nanoparticles at, 75,000 X magnification

3.7.3.7 Drug Release Study

Figure 7 exhibits the sustain drug release of ofloxacin –PHB nanoparticles at different time intervals. It was observed that the absorbance increased slowly with time interval. The initial absorbance was found to be 0.0 at 0 hr and after 30 days the absorbance was observed to be 3.99.

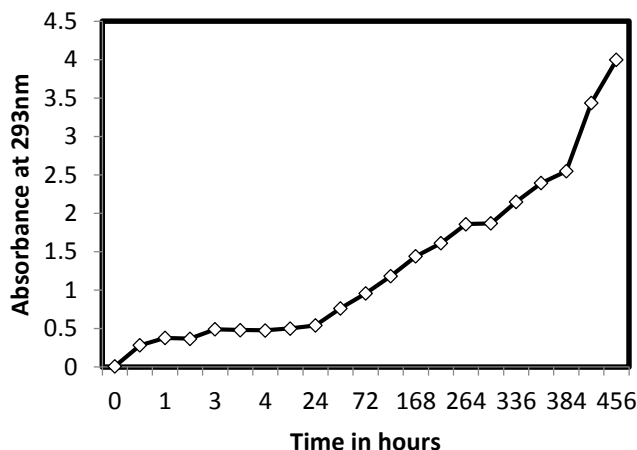


Figure 7: In vitro drug release study for PHB loaded with drug ofloxacin

4. DISCUSSION

Literature reviews that marine bacteria are the potential source of new bioactive compounds and has tremendous applications [8]. Microbes synthesize bioactive compounds under drastic and extreme situations. PHB producers were obtained from marine water samples. They were isolated and characterized using biochemical and molecular studies and were found to be *Bacillus pumilus* and *Enterobacter spp* [1]. These microbial cultures were directly used for the study. The microbes revealed the presence of PHB granules under phase contrast microscope. The cells cultivated in the fermentative medium using citrate as the sole source of carbon. PHB was extracted by sodium hypochlorite method and was quantified using crotonic acid as the standard. Due to the need of utilizing cheaper sources of carbon, agricultural waste was used. The agro waste which was cheaper and easily available was used for the study. Both the cultures were found to give increased PHB production of 24.12 % (*B. pumilus*) and 24.96 % (*Enterobacter spp*) on using banana peel extract powder. Whereas, for bagasse it was found to be 20.57% (*B. pumilus*) and 20.2% (*Enterobacter spp*). Banana peel is a rich source of carbohydrates and trace minerals whereas, bagasse is packed with simple reducing sugars. These simple reducing sugars enter the glycolytic pathway that increase the production of acetyl CoA. Two molecules of acetyl CoA conjugate to form acetoacetyl CoA which then undergoes series of biochemical reactions to form PHB [9]. Adwitiya P. *et al.*, 2009 obtained the highest PHB production with glucose after 48 h as it is the easily assimilable source of carbon that encourages bacterial production of PHB [10]. Getachew *et al.*, 2016 and Kulkarni *et al.*, 2015 reported increased PHB production using pre-treated bagasse while decreased PHB production on use of pre-treated banana peel due to less content of reducing sugars [11,3].

pH favours the product formation and metabolic reactions are susceptible to slight changes in pH [12] The maximum PHB production was obtained at neutral pH of 7 for both the microbial cultures.

The pH also affects the bioavailability of trace elements required for the metabolic processes. Flora *et al.*, 2010 obtained maximum PHB production by *Bacillus sphaericus* at a pH range of 6.5-7.5 [13]. Hamieh A. *et al.*, 2013 obtained maximum PHB for *Lactobacillus acidophilus* and *Bacillus thuringiensis* at pH 5.5. Panda *et al.*, 2006 reported it to be 8.5 for *Synechocystis* spp. PCC6803 [15].

For a microbial culture obtained from marine source will synthesize its bioactive compounds only in the environment from which it is obtained. The maximum concentration of sodium chloride was found to be 2% for *Bacillus pumilus* and 1% for *Enterobacter* spp. Mahitha and Jaya (2015) reported 2.5% NaCl tolerance for *Clavibacter michiganensis* M3 [17]. Arora *et al.*, 2006 suggested that NaCl balances the osmotic pressure in the salt tolerant bacterial cells which has resulted due to large storing capacity of PHB granules [18]. At the same time, NaCl decreases the free CoA concentration which reduces the inhibition of 3-ketothiolase, which catalyses the acetoacetyl-CoA formation from two molecules of acetyl CoA during PHB synthesis.

Ofloxacin is administered orally but has been reported to have the poor aqueous solubility of that gives rise to poor bioavailability. In addition, almost all of the oral ofloxacin formulations are available only as conventional, immediate-release tablets that require twice daily administration for consecutive days or weeks. [19-21] The repeated oral doses of ofloxacin over long time could result in nervous system and gastrointestinal system disorders. The in vitro drug release study indicated the PHB – ofloxacin nano biopolymer is a sustain release drug delivery system which continuously releases the medication over extended period of time after administration of single dose achieving *prolonged therapeutic effect*. Sustain release drug dosage forms provide better control of plasma drug level, less dosage frequency, less side effects, increased efficacy and constant drug delivery [18]. Sasikumar and Ayyasamy, 2015 obtained PHB based polymeric nanoparticles for controlled release of doxorubicin for cancer treatment [7].

5. CONCLUSION

PHB production is increased by utilizing banana peels as the source of carbon at neutral pH with 2% NaCl. The goal in designing sustained release drug delivery is to reduce the frequency of the dosing or to increase effectiveness of the drug by localization at the site of action, reducing the dose required or providing uniform drug delivery with increased efficacy and constant delivery. The drug loaded Ofloxacin - PHB nanobiopolymer existed as a form of sustained release drug delivery system. In future, such studies could be carried out for metabolically unstable and for drugs with short half life for which the nanobiopolymers could be utilized.

6. ACKNOWLEDGEMENT

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7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Effect of Whey on Protein, Carbohydrate and Chlorophyll Content of Wheatgrass

S. PATEL, N. BHANUSHALI, D. AIYA, DR. T. TIRODKAR*

Department of Biotechnology, V.G. Vaze College of Arts, Science and Commerce, University of Mumbai, Mumbai – 400081, India

ABSTRACT: Wheatgrass (*Triticum aestivum*) is a nutritionally rich plant and is gaining popularity among people day by day as a healthier substitute for morning beverages like canned fruit juices. On the other hand, whey is waste by product of cottage cheese production but it also contains important metabolites like proteins in high amounts. Considering the benefits of wheatgrass and whey, this research was carried to observe the effect of whey- when used as substrate along with soil - on the nutritional composition of wheatgrass. Wheat grains (*Triticum aestivum*) were grown in soil containing different concentrations (Control, 50% and 100%) of whey, an organic dairy waste that is thrown away after the production of cottage cheese from buffalo milk. The wheatgrass, after 9 days of growth were cut and extracted using 80% acetone as a solvent. Protein content was estimated using Biuret's method and separation of amino acids was done using Thin Layer Chromatography. Carbohydrate estimation was done using Anthrone method and separation of sugars was done using Thin Layer Chromatography, sugars were tested using various tests like Iodine test, Barfoed's test, Mucic acid test and Osazone test. Chlorophyll pigments were estimated using spectrophotometer and pigments were separated using Thin Layer Chromatography. TLC plates were observed under UV transilluminator. Three types of samples were used according to the concentration of whey in soil: Control, 50% whey, 100% whey. Of these 50% whey concentration wheatgrass has shown to have increased concentration of all the parameters under test i.e. proteins, carbohydrates and chlorophyll. Thus whey can be used at a household level as a nutrient enriching substrate in the soil used for growing wheatgrass

KEYWORDS: Wheatgrass (*Triticum aestivum*), Whey, Biofertilizer, Protein, Chlorophyll, Carbohydrates, Biuret test, Anthrone method, Spectrophotometry

***Corresponding Author: Dr. Tanuja Tirodkar**

Department of Biotechnology, V.G. Vaze College of Arts, Science and Commerce, University of Mumbai, Mumbai – 400081, India

Email Address: tirodkartanuja@gmail.com

1. INTRODUCTION:

Whey (the liquid left after milk curdling), a by-product of cheese production, is a greenish-yellow solution composed of water, lactose, proteins and minerals that represents 85–90% of the milk volume[1]. Whey was deemed a waste by the dairy industry for decades. The effluent caused major disposal issues, due to its high organic matter and resultant high biological oxygen demand. Whey has been quantified to contain 15–20 % of total milk proteins and that is too big an amount to let go. Whey protein is a globular protein with main components as β -lactoglobulins (35–65 %) and

α -lactalbumins (12–25 %). The minor ingredients include immunoglobulins (8 %), serum albumins (5 %) and lactoferrin (1 %) Whey protein is a rich source of branched-chain amino acids (leucine, isoleucine, and valine), essential amino acids (cysteine) and peptides as well. A broad range of functionality has been assigned to whey protein and its derivatives, such as reduction of oxidative stress, promotion of muscle growth and lean body mass, appetite suppression, hypoglycemia, cardiovascular risk mitigation, phenylketonuria management and protection from ultraviolet (UV) radiation.

Wheat, (*Triticum* species) a cereal grass of the Gramineae (Poaceae) family, is the world's most edible grain cereal-grass crop. Nowadays, researchers have known Wheatgrass as a nutrient-rich type of young grass in the wheat family, and are many times richer in levels of vitamins, minerals and proteins as compared to seed kernel, or grain products of the mature cereal plant. At present, wheatgrass is quickly becoming one of the most widely used supplemental health foods and is available in many health food stores as fresh produce, tablets, frozen juice, and powder. Wheatgrass provides a concentrated amount of nutrients, including iron; calcium; magnesium; amino acids; and vitamins A, C and E and large amounts (70%) of chlorophyll. Some proponents tout wheatgrass as a treatment for cancer, ulcerative colitis and joint pain, and also serve as antioxidant. It has been suggested that wheatgrass has greater nutritional value than several everyday foods, and ingesting wheatgrass is comparable to eating a large amount of vegetables [2]. An attempt to incorporate whey as a biofertilizer in the cultivation of wheatgrass was executed to evaluate its effect on the protein, chlorophyll and carbohydrate content of wheatgrass.

2. MATERIALS AND METHODS

2.1.Cultivation of wheatgrass using 3 different concentrations of whey [3]

The wheatgrass used in this research was grown indoors until required. The whey water used was obtained from homemade cottage cheese preparation done using buffalo milk. 3 Plastic trays (10" circumference, 2.5 cm height) were filled with upto 2" of regular potting soil and labelled as control tray, low conc. tray and high conc. tray. Control tray was prepared by adding 100 mL of distilled water to the soil. Low conc. tray was prepared by adding 50 mL of whey diluted upto 100 mL with distilled water to the soil. High conc. tray was prepared by adding 100 mL whey to the soil. Wheat grains (unpolished) which were soaked overnight in water were evenly distributed in each tray keeping the number of seeds added in each tray similar. Seeds were pressed lightly to partially submerge the seeds in the soil. The trays were kept in a partially covered balcony and watered regularly. They were allowed to grow for 9 – 10 days till wheatgrass reached a height of upto 6 – 7 inches.

2.2.Extraction of wheatgrass [4]

Fully grown wheatgrass (6 -7 inches of height) was cut with the help of a clean pair of scissors from each tray and it was recovered in 3 different zip lock bags respectively as control, low conc. and high conc. Grass was kept at 37°C overnight to dry. It was coarsely powdered using a mixer and was used for extraction. Extraction was performed using 80% acetone (extraction ratio – 1:75) using a mortar and a pestle. Grinding was continued until discoloured grass particles appeared.

This mixture was filtered through whatman filter no. 41. The filtrate was then centrifuged twice for 15 minutes each at 6000 rpm. The filtrate was used for further analysis. The extracted sample was refrigerated until use.

2.3. Estimation of protein content

A. Protein Estimation of Control, Low & High Whey Concentration Wheatgrass Extracts [5]
All three samples were subjected to Biuret test of protein analysis which detects the presence of peptide bonds by formation of violet-coloured complexes in alkaline solution. Protein concentration of test samples (Control, low whey & high whey) was obtained using standard Bovine Serum Albumin (BSA) of 2mg/ml concentration. Prepare the tubes as per Table 1. Incubate the tubes at 37°C for 30 minutes & measure absorbance using a spectrophotometer at 540nm. Prepare a standard curve of absorbance versus milligrams protein (or vice versa), and determine amounts of unknown from the curve.

Sr. No.	Protein Concentration (mg)	Standard protein (ml)	Distilled water (ml)	Biuret reagent (ml)
Blank	0.0	0.0	2.5	2.5
1	0.2	0.1	2.4	2.5
2	0.4	0.2	2.3	2.5
3	0.6	0.3	2.2	2.5
4	0.8	0.4	2.1	2.5
5	1.0	0.5	1.9	2.5
6	1.2	0.6	1.8	2.5
7	1.4	0.7	1.7	2.5
8	1.6	0.8	1.6	2.5
9	1.8	0.9	1.5	2.5
10	2.0	1.0	1.4	2.5
Control	-	-	1.5	2.5
Low whey	-	-	1.5	2.5
High whey	-	-	1.5	2.5

Table 1: Absorbance values of Standard and Test samples

B. Thin Layer Chromatography for detection & isolation of amino acids

A solvent chamber using butanol: acetic acid: water in 80:20:20 ratio as the solvent was prepared and saturated for 30 minutes. Samples were spotted on the TLC plates at a distance of 1.5 cm from the bottom using a capillary tube. The plates were placed in the TLC solvent chamber till the mobile phase raises upto 3/4th of the plate. The solvent front was marked using a pencil. Plates were dried using a hair dryer. The plates were sprayed with ninhydrin reagent and dried at 105°C for 5 minutes. Spots were marked and Retardation factor (Rf) were calculated.

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2.4. Estimation of carbohydrate content [6]

A. The method used for estimation of carbohydrates was anthrone method.

The required reagents were:

- a. Anthrone reagent – 200 mg of anthrone reagent in 100 mL conc. H_2SO_4 .
- b. Standard glucose solutions:
 - Stock standard – 100 mg glucose in 100 mL D/W.
 - Working standard – 10 mL stock standard solution in 100 mL D/W.
 - Estimation tubes were prepared according to the following table.

Tube no.	Working standard (mL)	Distilled water (mL)	Test solution (mL)	Anthrone reagent (mL)
1 (B)	-	1.0	-	4.0
2 (S)	0.5	0.5	-	4.0
3 (T ₁)	-	0.5	0.5 (control extract)	4.0
4 (T ₂)	-	0.5	0.5 (low conc. extract)	4.0
5 (T ₃)	-	0.5	0.5 (high conc. extract)	4.0
Keep the tubes in boiling water bath for 10 minutes, cool to room temperature and take the absorbance of each tube using a spectrophotometer at 620 nm.				

Key: B = blank, S = standard, T₁ = control tube, T₂ = low conc. tube, T₃ = high conc. tube.

B. Detection of types of sugars

The following test was performed to detect the types of sugars in the wheatgrass sample.

OSAZONE TEST [7]. (Test for presence of ketoses and aldoses).

0.5 g of phenylhydrazine hydrochloride was added to 0.1 g of sodium acetate and 10 drops of glacial acetic acid was added and to this mixture about 5 mL of sample solutions were added. Three such tubes were prepared for three sample solutions – control, low conc., high conc. All the three tubes were kept in boiling water bath for half an hour. After half an hour the tubes were allowed to cool down slowly. From each tube one drop of solution was suspended on a clean slide and the drop was allowed to dry. These dried slides were observed under microscope to check for the formation of crystals of osazone.

C. Thin layer chromatography of carbohydrates [8]

3 mL of ethanol was added to 1 mL of wheatgrass samples. Three such tubes were prepared for three test samples. The samples were centrifuged for 15 minutes at 6000 rpm and the supernatant of each of the three samples were separated and spotted on activated TLC plates. Standard sugar

solutions of glucose, fructose, mannose and sucrose were also for comparison. After drying, it was allowed to run in a saturated chamber of solvent – ethyl acetate: isopropanol: water: pyridine (26:14:7:2). After drying, the plate was sprayed with developing reagent – aniline-diphenylamine (prepared fresh by mixing 5 volumes of 10 g/L aniline and 5 volumes of 10 g/L diphenylamine in acetone with one volume of 85% phosphoric acid). The plate was kept for colour development at 100°C and then viewed under UV transilluminator at 366 nm.

2.5. Estimation of chlorophyll content.

A. Estimation of sample by Spectrophotometry

Spectrophotometer is an important instrument used in many biochemical experiments. In this study spectrophotometer was used to find absorbance values of pigments in test samples. The analytical determination was performed with visible spectrophotometer at the following wavelengths 662, 645 for chlorophyll a and chlorophyll b respectively and 470nm for carotene (according to extraction solvent)

Chlorophyll a = $11.75 A_{662} - 2.350 A_{645}$

Chlorophyll b = $18.061 A_{645} - 3.960 A_{662}$

Carotene = $1000 A_{470} - 2.270 \text{ Chlorophyll a} - 81.4 \text{ chlorophyll b} \times 227$

Thin layer chromatography of chlorophyll pigments:

All the three samples of wheatgrass were applied on activated TLC plate as three spots and they were allowed to dry completely. The TLC plate was kept in a saturated chamber of solvent – petroleum ether: isopropanol: distilled water and it was allowed to run upto 3/4th area of the plate. The solvent front was marked and the plate was allowed to dry. The R_f value of each sample was calculated by using the formula: $R_f = \text{distance travelled by solute} / \text{distance travelled by solvent}$.

3. RESULTS AND DISCUSSIONS

Concentration of whey.	Protein content (mg/mL)	Carbohydrate content (g/L)	Chlorophyll content		
			Chlorophyll a	Chlorophyll b	Carotenoids
Control	2.34	0.0993	10.59	4.61	3.50
50% (v/v)	4.86	0.1109	31.15	21.79	7.6026
100% (v/v)	3.44	0.1048	26.69	18.44	9.52

Table 2: Protein, Carbohydrate and chlorophyll content in all the three wheatgrass samples.

Amino Acid	Standard Rf	Observed Rf
Glutamic Acid	0.25	0.275
Valine	0.4	0.35 / 0.375
Tyrosine	0.42	0.425 / 0.45
Lysine / Leucine	0.58	0.6

Table 3: Rf values of separated amino acids.

	Chlorophyll a	Chlorophyll b	Carotenoids
Control	10.59	4.61	3.50
Low	31.15	21.79	7.6026
High	26.69	18.44	9.52

Table 4: Chlorophyll content in wheatgrass samples.

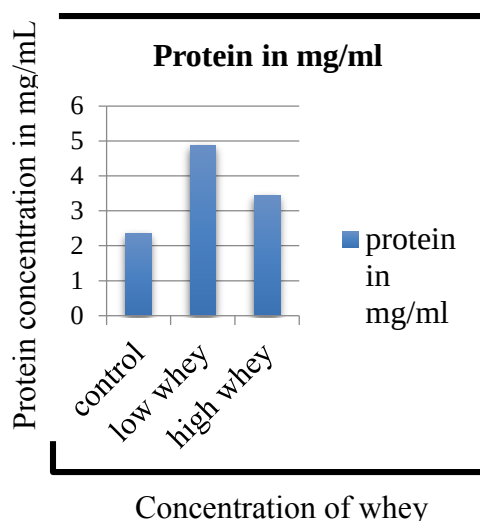


Figure 1: Graphical representation of protein content in wheatgrass samples.

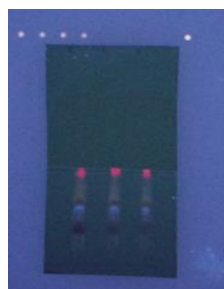


Figure 2: TLC plate of amino acid separation.

4. DISCUSSIONS

The effect of whey protein on the protein content of wheatgrass is evident in the low as well as high whey concentration extracts. Low whey content plant show an increased protein content when compared with the control and high whey plants.

TLC of the 3 samples show distinct bands of amino acids in UV as well as visible light. This confirms the presence of amino acids like glutamic acid, valine, tyrosine, lysine & leucine in wheatgrass.

The carbohydrate content is highest in the low conc. Sample followed by high conc. sample which is followed by control sample.

Yellow coloured needle shaped crystals were seen in the control sample. Thus it suggests the presence of glucose and fructose.

Needle shaped crystals were observed in the 50% (v/v) whey sample of wheatgrass. Thus this

suggests the presence of sugars like glucose, fructose, aldoses and ketoses.

Mushroom shaped crystals were seen in the 100% (v/v) whey wheatgrass sample. Such crystals suggest the presence of lactosazone. Thus we can infer that sugars like lactose, ketoses and aldoses were present in the sample.

TLC separation of sugars was done but the bands were not clearly distributed. Thus this shows that indeed sugars are present in all the concentrations of sample, but they might be present in low concentrations as they are not giving distinct bands.

All the chlorophyll pigments were also seen to be the highest in the low conc. whey sample when compared to control and high conc. whey sample. The only exception to this was carotenoids.

Thus overall the low whey conc. sample was seen to be the most effective for the growth as well as was found to be responsible for increase in the protein, carbohydrate as well as chlorophyll content. As the results found, low whey content when added, that is 50mL whey in 100mL water, outranked high concentration whey (100 % whey) as after a certain amount of whey concentration it shows a deteriorating effect on the sample taken. This effect of high whey concentration on protein, chlorophyll decreases due to increase in acidity of the plant and hence low whey content turns out to be the most beneficial concentration when checked on the 3 parameters i.e. protein, carbohydrate & chlorophyll. This research work indirectly has health-care as well as environmental benefits on our day-to-day lives.

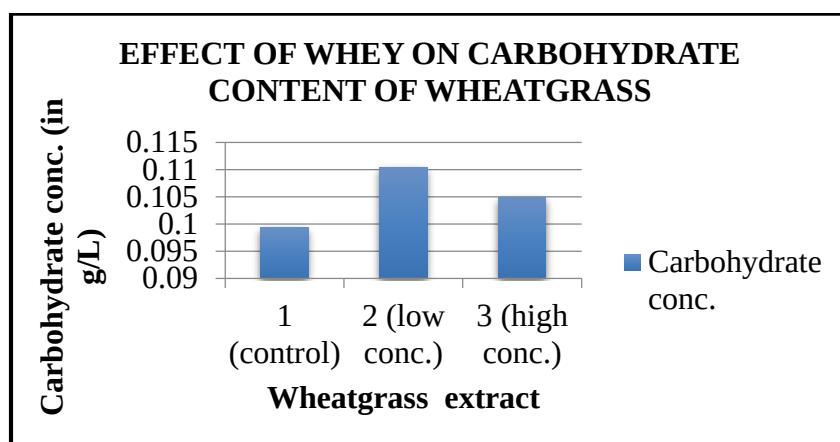


Figure 3: Graphical representation of carbohydrate in wheatgrass samples

5. CONCLUSION

Effect of whey, a dairy waste, was studied on the protein, carbohydrate & chlorophyll content of wheatgrass. As per the results, there is a considerable increase in all the 3 parameters i.e. protein, carbohydrate & chlorophyll when incorporated with 50% whey. (50mL whey in 100mL water). The effect of high concentration whey (100 % whey) on wheatgrass was considerably higher than control but lower than 50% whey. This may be attributed to the low pH of whey (≤ 5.1). This acidic pH restricts the plant growth and high concentration of whey might also reduce nutrient uptake by wheatgrass. Whey was deemed a waste by the dairy industry for decades. The effluent caused major disposal issues, due to its high organic matter and resultant high biological oxygen demand.

This suggests that there are future applications of whey being used as a biofertilizer to enhance the nutrient content of wheatgrass & this may reduce the potential pollutant nature of whey. Further research can be done on improving the nutrient uptake by the wheatgrass. This method can successfully be used at a household level to improve the nutritional value of the wheatgrass and this can included as a part of a daily diet as a means to boost immunity especially for diseased individuals. Dried powder of such a plant can be marketed. Thus, whey-infused wheatgrass can be used as it is known to help minimize fatigue, improve sleep, increase strength, naturally regulate blood pressure and blood sugar, support weight loss, improve digestion and elimination, support healthy skin, teeth, eyes, muscles and joints, improve the function of our heart-lungs and reproductive organs, heal ulcers and skin sores, slow cellular aging, improve mental function, and is beneficial in arthritis and muscle cramping. It is proven to be beneficial under various conditions, such as anaemia, diabetes, cancer, eczema, constipation, kidney swelling, and common cold. Thus, it should be made part of daily dietary intake in order to explore its maximum benefits.

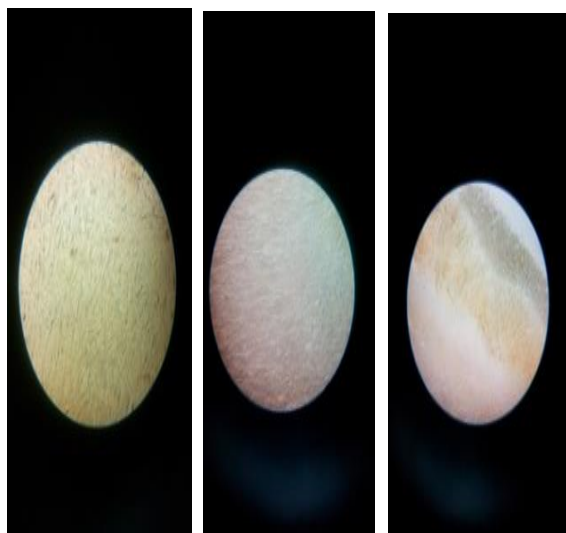


Figure 4: Osazone test results in control sample, low conc. sample and high conc. sample respectively from left to right.

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7. CONFLICT OF INTEREST

There is no conflict of interest.

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Screening and Isolation of pigment from *Rhodotorula mucilaginosa* from natural source (curd)

B. MHATRE *, N. KAKRANIA

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Synthetic pigments are being used in various marketable products but they are hazardous, carcinogenic and non-biodegradable. The utilization of natural pigments in food, dyes, cosmetic and pharmaceutical manufacturing processes has increased in recent years. Microbial pigments are stable, biodegradable, environment friendly and have numerous benefits. The cultivation of microbes is easy and fast in the cheap culture medium and independent from weather conditions. Microbial pigment has the potential for various industrial applications. In our present study we have isolated organism from curd sample which is a natural and easily available source. The organism was identified by biochemical test, morphological characterization and rDNA sequencing (funits). The isolated organism was identified as *Rhodotorula mucilaginosa* and its pigment was extracted. The carotenoid pigment was identified using GC-MS and UV spectroscopy. Its toxicity was checked with two gram-positive and gram-negative bacteria commonly found in the human gut flora using agar well diffusion method and it was found to be non-toxic.

KEYWORDS: pigment extraction, rDNA sequencing (funits), *Rhodotorula mucilaginosa*, carotenoid, GC-MS, UV spectroscopy.

***Corresponding Author: Dr. Bhakti A Mhatre**

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: bhaktiamhatre@gmail.com

1. INTRODUCTION

All body cells are constantly exposed to internal and external oxidants. In recent years, the intensification of air pollution, UV radiation, smoking and improper diet has increased the amount of these oxidants alarmingly and ultimately leads to incidence of various disease and disorders such as cancer, cardiovascular disease and cataracts in human [1].

The microbial contamination is one of the leading causes of food spoilage worldwide [2]. The organisms that cause the microbial contamination can also be used in a way that is adventitious to humans.

Colours provide attracting appearance to marketable products such as food products, textiles, and pharmaceutical products. Pigments from natural sources have been obtained since long time ago, and their interest has increased due to the toxicity problems caused by the synthetic pigments [3]. More research focused on the use of agro-industrial wastes, high carotenoids-producing yeast through metabolic engineering strategies, bioreactor design and control strategies for microbial carotenoids production are necessary to cover the worldwide demand by poultry, cosmetic, food,

feed, beverages and other industries [4].

There are many limitations of synthetic pigments. The precursors, used in the production of synthetic pigment, may have many carcinogenic, hazardous effects on the workers [5]. The wastes produced during the process too can be harmful. They are non-environment friendly and non-biodegradable. To counter these hazardous effects of synthetic colorants, there is worldwide interest in process development for the production of pigments from natural sources [6]. Plants and microorganisms are the two major sources of natural pigments. Yet the natural pigments from plants also have drawbacks such as instability against light, heat or adverse pH, low water solubility and non-availability throughout the year [7]. Hence the microbial pigments are of great interest owing to the stability of the pigments produced and the availability of cultivation technology [8].

Microbial pigments have numerous beneficial properties like anticancer, antiproliferative, immunosuppressive, antibiotic, biodegradability etc. Many microorganisms, including bacteria, fungi, yeast and mould etc. are employed for the industrial production of various pigments by using fermentation technology [9]. Hence, microbial pigment production is now one of the emerging fields of research to demonstrate its potential for various industrial applications [10].

Rhodotorula species are ubiquitous saprophytic yeasts that can be recovered from many environmental sources, including humans, animals, and a large variety of foods and beverages [11]. In keeping with its capability to survive and grow in many unfavourable conditions, *Rhodotorula mucilaginosa* possesses valuable biotechnological features, including copper biotransformation, the production of biosurfactants, and high yields of unsaturated fatty acids and carotenoids [12].

Genus *Rhodotorula* produces carotenoid pigments, giving a salmon-pink to coral-red colour to the colonies and presenting only spheroidal to oval budding cells without the rudimentary formation of hyphae [13].

Despite the lack of utilizing lactose, the main sugar present in milk, as carbohydrate, the ability to grow rapidly at low temperatures, contributes to the presence of *Rhodotorula* species in dairy products like butter, yogurt, soft cheeses, and cream. Because the species produce extracellular proteases and lipases, it is likely that they may cause spoilage [14]. *Rhodotorula mucilaginosa* is predominant in yogurts and at lower numbers in cheeses, butter, and cream in which they usually are associated with pink spots on the surface [15].

The objective of this study was to extract the pigment from *Rhodotorula mucilaginosa* which was isolated from curd and grown on potato dextrose broth. The characterization of the organism was done through various morphological and biochemical tests like catalase test, urease test, oxidase test, carbohydrate fermentation of glucose, sucrose, lactose, xylose, raffinose and starch. The characterization of the pigment was made using UV-V spectrophotometer and GC-MS. The isolated organism was tested for toxicity if any with antibacterial test for human gut flora.

This study aims at introducing bacterial pigments as an alternative to synthetic dye. In this study, the pigment is isolated and extracted from an organism that is from natural sources which are easily available and is non-toxic to human.

2. MATERIALS AND METHOD

2.1.Isolation of microorganism

The organism was isolated from curd that was kept in the refrigerator for two weeks. Potato dextrose agar was used to maintain the yeast culture. The culture was stored in a refrigerator at 4°C.

2.2.Maintenance of the culture

Potato dextrose agar (PDA) was used for the cultivation and isolation of yeast. PDA enabled healthy growth and pigmentation for a wide variety of fungal taxa.

2.3.Morphological characterization

The cells were examined for their appearance such as surface, colony shape, colour, elevation, margin, cell shape, gram staining, motility test and biochemical characterization.

2.4.Molecular identification of bacteria

Funits use the internal transcribed spacer (ITS2) region, a commonly sequenced fungal DNA region. It refers to the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome. It is used because it has more variation between closely related species than other DNA regions such as D2.

The isolated organisms were sent to Charles River Laboratories, CBD Belapur. Genotypic methods, such as comparative sequencing of the ribosomal DNA ITS2 region in fungi, have been proven to be the most accurate and reproducible methods for identifying unknown organisms. The technology is inherently stable, which allows for reproducible data for identification.

2.5.Antibacterial test

A loop of isolated organism is used to inoculate PDA plates to make a lawn. Four wells were made using a 6 mm cork borer. 100µl of nutrient broth containing was poured into these wells, incubate the plates at 37°C overnight and record the zones of inhibition [16].

The antimicrobial activity was carried out by well diffusion method against the test organisms such as *S. Aureus*, *Sarcina lutea*, *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia*, *S. pyogenes*, *Shigella sonnei*, and *Bacillus subtilis*. The nutrient agar plates were prepared and previously seeded with the test organism in each plate. The wells were cut by using a sterile cork borer. 100µl of culture filtrate was added in each well. The diameters of inhibition were determined after 24 hr of incubation at 37°C. The experiment was performed twice to minimize the error.

2.6.Extraction of pigment

Exposure of the extract to heat, light, and oxygen should be minimized throughout the extraction procedure. The wet cells were collected by centrifuging the growth medium at 10000 rpm for 10 min and washed twice with distilled water [16]. Then 1 ml of ethanol was added to the cells and the tube was vortexed. The mixture was transferred to a 50-ml beaker. It was dried in oven at 40°C until the ethanol completely evaporated. The dried powder was scrapped off the beaker and crushed

with a mortar and pestle. The powder was transferred to a 1ml eppendorf and mixed with 1 ml acetone as a solvent. The supernatant was collected by centrifuging the eppendorf at 10000 rpm for 10 minutes [17, 18].

2.7. Identification of pigment

The identification of the pigment was done by UV spectroscopy. Most of the carotenoids exhibit absorption in the visible region of the spectrum. Between 400 and 500 nm. Absorbance measurements can be used to quantify the concentration of a pure (standard) carotenoid or to estimate the total carotenoid concentration in a mixture or extract of carotenoids in a sample [17].

2.8. GC-MS

The pigment in acetone solvent was sent to SAIF IIT Bombay, Powai. The GC-MS used was Agilent 7890 with fid detector, head space injector and combipal autosampler with a mass range of 10-2000 amu and a mass resolution of 6000.

3. RESULTS

3.1. Isolation of microorganism

The isolated organism was observed with help of light microscope. The organism was found to be gram positive, cocci shaped, occurring in small clusters. Hanging-drop wet method showed that the isolated organism was non-motile.

3.2. Biochemical tests

Biochemical tests showed presence of bubbles for catalase test indicating that the isolated yeast is catalase positive and could mediate the decomposition of H_2O_2 to produce O_2 . Catalase is found in all yeasts and it is an important enzyme as it protects the cell from oxidative damage by reactive oxygen species (ROS) [19].

Similarly, Oxidase, Urease and carbohydrate fermentation test were found to be positive. was positive as there was no change in the colour [19,20]. Table 1 shows that the isolated yeast could ferment glucose, sucrose, xylose and raffinose, but not starch and lactose. No air bubbles in the Durham tube indicate no gas production occurred.

It was because of the ability to ferment glucose that the organism can grow in dairy products. Isolated strain presented physiological, biochemical and morphological characteristics that fit the standard description of *Rhodotorula* species [21].

3.3. Molecular identification of bacteria

The accugenix funits identified the organism as *Rhodotorula mucilaginosa*. The sequencing technique uses the internal transcribed spacer (ITS2) region, a spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome. The internal transcribed spacer (ITS) regions of the ribosomal operon have been used for fungal systematics and classification. There are two ITS regions in the fungal rRNA operon.

Components and tests	Result
Catalase	Positive
Oxidase	Positive
Glucose	Positive
Sucrose	Positive
Lactose	Negative
Xylose	Positive
Raffinose	Positive
Starch	Negative
Urease	Positive

Table 1 Biochemical characteristics of the isolated organism

3.4.Antibacterial test

The isolated organism didn't have antibacterial activity against all the gram-positive and gram-negative organisms used. No inhibition zone was seen against *S. aureus*, *Sarcina lutea*, *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia*, *S. pyogenes*, *Shigella sonnei* and *Bacillus subtilis*. These organisms are found in human gut and play a pivotal role in direct inhibition of pathogens, development of enteric protection and immune system and metabolism. The isolated organism has no toxic effect on the human gut flora Fig 1 and 2.



Figure 1: Isolated colonies of *Rhodotorula mucilaginosa* grown on nutrient agar plate.

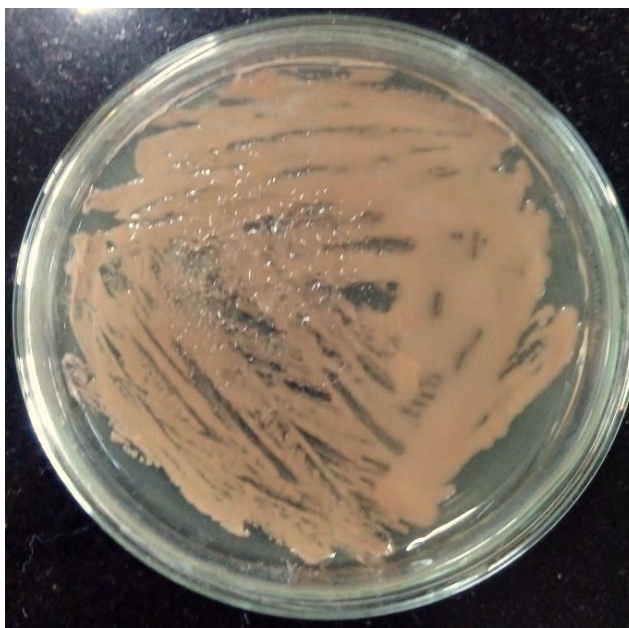


Figure 2: Isolated colonies of *Rhodotorula mucilaginosa* grown on potato dextrose agar plate.

3.5.Extraction of pigment

The pigment was extracted from *Rhodotorula mucilaginosa* and dissolved in acetone for further analysis Fig 3.



Figure 3 Extracted pigment from *R. mucilaginosa* cells dissolved in acetone on the right

The highly unsaturated carotenoid is prone to isomerization and oxidation [22]. Heat, light, acids, and adsorption on an active surface promote isomerization of trans carotenoids, their usual configuration, to the cis forms. This results in some loss of colour and provitamin A activity [23].

3.6.Identification of pigment

GC-MS combines the advantages of both techniques the high resolving power and the speed of analysis of GC with the qualitative and quantitative analytical capabilities of the MS down to the

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ppb level.

Figure 4 shows the complete chromatogram of the pigment dissolved in acetone. It is a plot along an x-axis and y-axis of the total ion current intensity vs. retention time respectively obtained from a chromatography experiment coupled with mass detection. In most forms of mass spectrometry, the intensity of ion current measured by the spectrometer does not accurately represent relative abundance, but correlates loosely with it. Therefore, it is common to label the y-axis with arbitrary units.

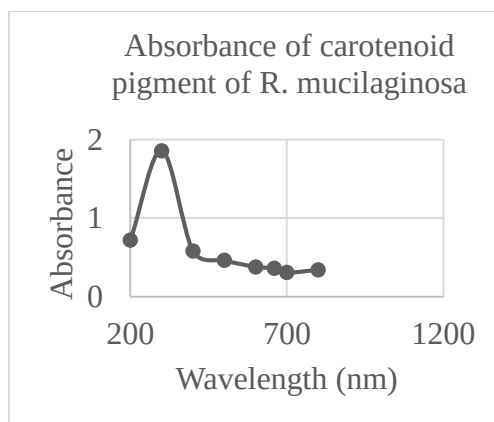


Figure 4: Complete chromatogram of the pigment dissolved in acetone

4. DISCUSSION

Molecular identification of bacteria

The first, ITS1 found between the 18s and 5.8s rRNA genes. The second, ITS2, is located between the 5.8s and the 28s rRNA genes. The entire rRNA operon is transcribed, since the ITS sequences are important enough as spacer regions to be maintained by the cell, but not used for any functional purpose, they accumulate mutations at a faster rate than the 5.8s, 18s, and 28s rRNA genes. This slightly increased rate of accumulated mutations allows the ITS sequences to provide an improved level of resolution as compared with the d2 sequence. It is therefore customary among the fungal phylogeneticists to sequence the entire stretch of ITS1-5.8s-ITS2 to use in fungal classification. However, for the routine identification purposes, the use of ITS2 alone is usually sufficient.

The ITS region is more often advocated than cautioned against as a vector for species identification in fungi, but these reports are typically based on subsets of fungi, often at the family level or lower. The picture emerging from joint analysis of all available fungal sequences should be highly indicative of the performance of the ITS as a barcode region in the fungi. ITS region will remain a mycological cornerstone for a long time to come [24]. The isolated organism showed maximum identification to *Rhodotorula mucilaginosa*.

Antibacterial test

There have been cases of infection caused by *Rhodotorula mucilaginosa* in immunocompromised patients. But it seems more likely that the organism is an opportunist that takes advantage of immunocompromising conditions, indwelling devices, and exposure to broad-spectrum antibiotics.

to colonize and infect at-risk patients Fig 5 and 6. Antibiotics and exposure to cytotoxic agents may increase gastrointestinal colonization and intestinal mucosa damage. Due to the small number of published cases, it is not possible to recommend a management of this type of infection [25].

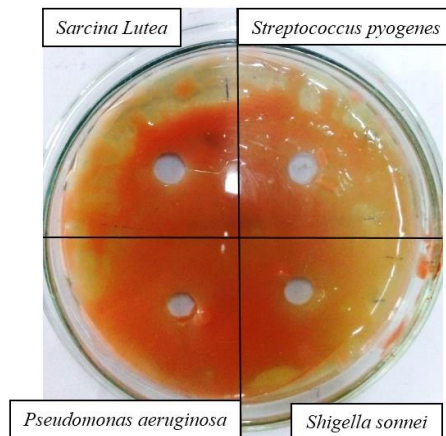


Figure 5: Plate showing antibacterial activity of *Rhodotorula mucilaginosa* by agar well diffusion method against *Sarcina Lutea*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and *Shigella sonnei*.

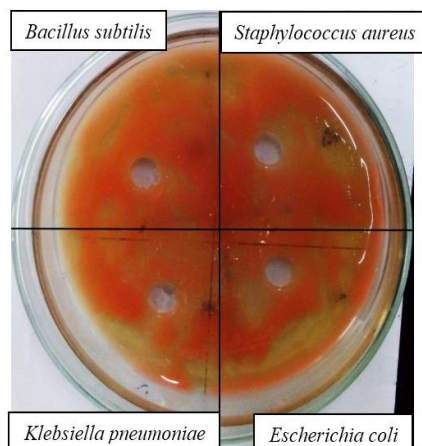


Figure 6: Plate showing antibacterial activity of *Rhodotorula mucilaginosa* by agar well diffusion method against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli*.

Extraction of Pigment

Oxidative degradation, the principal cause of extensive losses of carotenoids, depends on the availability of oxygen and is stimulated by light, enzymes, metals, and co-oxidation with lipid hydroperoxides. Thus, total loss of colour and biological activities are the final consequences. Conditions necessary for isomerization and oxidation of carotenoids exist during preparation, processing, and storage of food. Thus, retention of naturally occurring or added carotenoids in prepared, processed, and stored foods is an important consideration [26]. Carotenoids are also

subject to isomerization and oxidation during analysis, and preventative measures must be taken to guarantee the reliability of analytic results [27]. Leaving carotenoids in solvents such as cyclohexane, dichloromethane, diethyl ether, and acetone can lead to substantial degradation [28]

Identification of pigment

The UV absorption spectrum is still the first diagnostic tool for the identification of pigments. Both the position of the absorption spectrum and the shape of the spectrum give a preliminary characterization. Because of their long-conjugated double-bond system, the carotenoids absorb light strongly and exhibit intense main absorption bands in the visible or in some cases, UV region Fig 7 [29]. UV spectrum gives a lot of information about the pigment. The wavelength of maximum absorption provides structural information, specifically about the length of the chromophore Fig 8.

The choice of solvent is also very important as it could influence the absorbance and the structure of the chromophore. Of all the solvents, acetone causes the minimum deviation of just 2-5 nm, while solvents like chloroform or dichloromethane cause a deviation of about 10-20 nm. Also, petroleum, diethyl ether, benzene or hexane which are used as a solvent for carotenoid extraction have low polarity which influences the spectral fine structure [30].

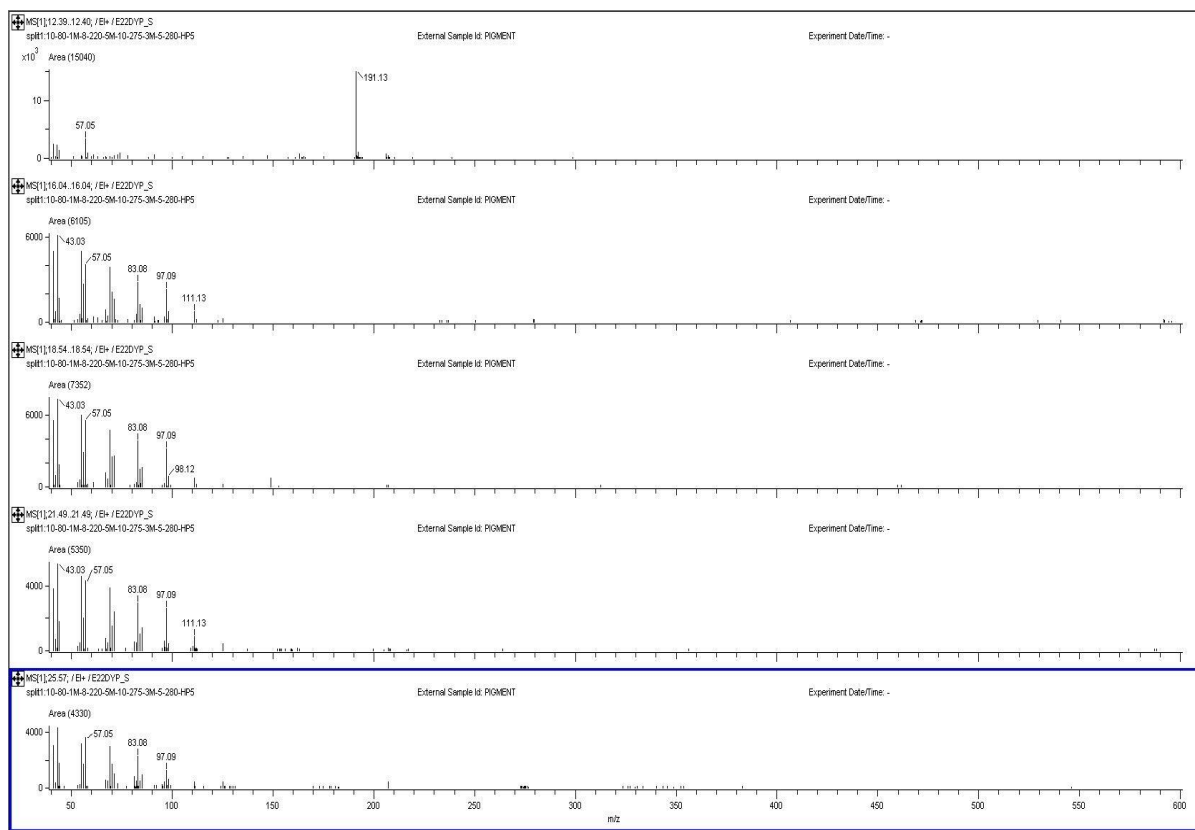


Figure 7: Spectra in acetone of the carotenoid extract obtained from *R. mucilaginosa*, with λ_{max} .

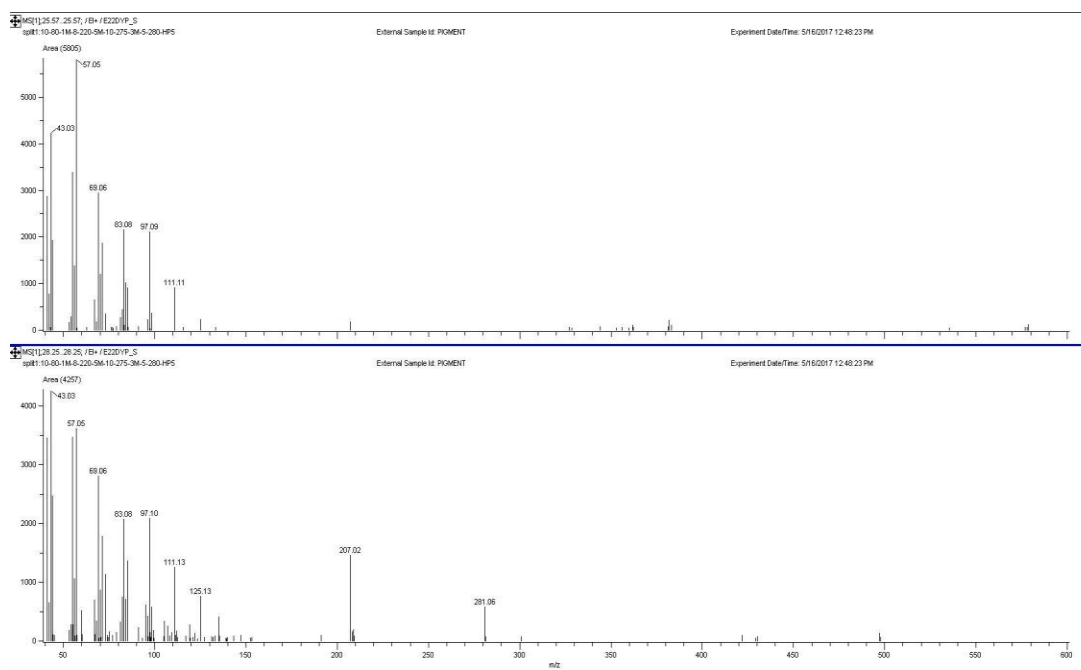


Figure 8: Mass spectrum of pigment dissolved in acetone

GC-MS

Mass spectroscopy result for peaks that have been identified using the NIST database. The NIST Mass Spectrometry Data Center, develops evaluated mass spectral libraries. These libraries are intended to assist compound identification by providing reference mass spectra for GC/MS (by electron ionization).

The sample analysed by the GC-MS contained carotenoid pigment in acetone solvent. Carotenoids usually found in *Rhodotorula* species is Tolruene, Torularhodin, gamma-carotene and beta-carotene with a molecular mass of 534.872 g/mol, 564.854 g/mol, 536.888 g/mol and 536.888 g/mol respectively [31].

The first peak in the above chromatogram is the peak of acetone as it was the solvent and therefore it would have the highest peak.

The molecules identified by the NIST database are Oxirane, Phenol, 2,4-bis-(1,1-dimethylethyl), Tetradecyl trifluoroacetate, Oxalic Acid, Allyl Decyl Ester and 1-Docosene with a molecular mass of 44.053 g/mol, 278.511 g/mol, 310.3955 g/mol, 270.369 g/mol and 308.594 g/mol respectively. This potentially proves that the molecules detected are the fragments of the different types of carotenoids present in the pigment extract Fig 9.

The molecules identified by the NIST database are Oxirane, Phenol, 2,4-bis-(1,1-dimethylethyl), Tetradecyl trifluoroacetate, Oxalic Acid, Allyl Decyl Ester and 1-Docosene with a molecular mass almost identical peak [1]. The benzene ring can be easily seen in the MS as it hasn't degraded but its functional groups have reacted. The carbon chain has been cut from several places to give shorter carbon chain.

It is also possible that the carotenoids suffered from slight degradation due to the acetone solvent. As carotene is destroyed when dissolved in acetone or petroleum ether and exposed to light. The

reaction is a function of time, the longer the exposure to light, the greater the loss of carotene [32].

5. CONCLUSION

The bacteria were successfully isolated from a natural source (curd) and identified as *Rhodotorula* species using gram staining, motility and biochemical tests. The identity of *R. mucilaginosa* was further confirmed using funits rDNA sequencing.

The organism can be obtained and grown easily with high amount of glucose. *Rhodotorula mucilaginosa* can be used as a pigment producing yeast to give a red or salmon colour to a food or drug. Some research is needed to analyse on how the organism can be grown on a large industrial scale and processed for a high production of the pigment.

In recent years, there has been an increased consumer knowledge of negative impact of synthetic colorants on health. Therefore, researchers are looking for new producers of natural dyes. *R. Mucilaginosa* are capable of synthesizing carotenoids which have not been detected in foods, and probably of this, their effects on the human health have not been investigated and described yet. However, considering their chemical structure and properties, it seems clear that these two substances can be used as food additives. *R. Mucilaginosa* yeasts have great potential for industrial applications.

Even though the biosynthesis of carotenoids by *Rhodotorula* yeasts is well known, their industrial use is restricted mainly because of poor information on biosynthetic regulating mechanisms and lack of studies

However, it is necessary to conduct further research for the industrialization of this yeast and its products.

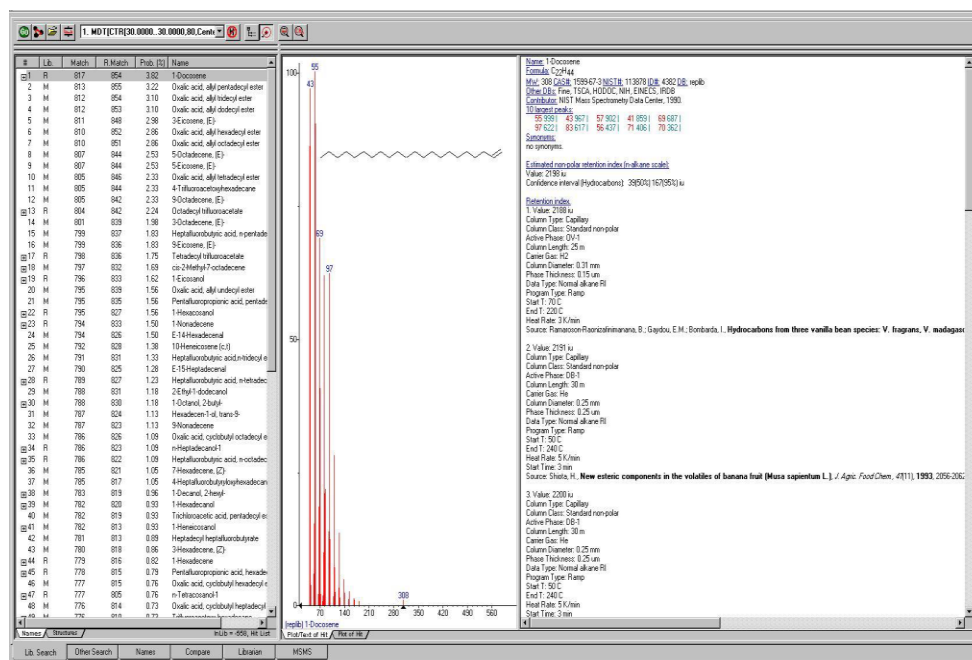


Figure 9: NIST Mass Spectral Library displaying 1-Docosene as the unknown peak

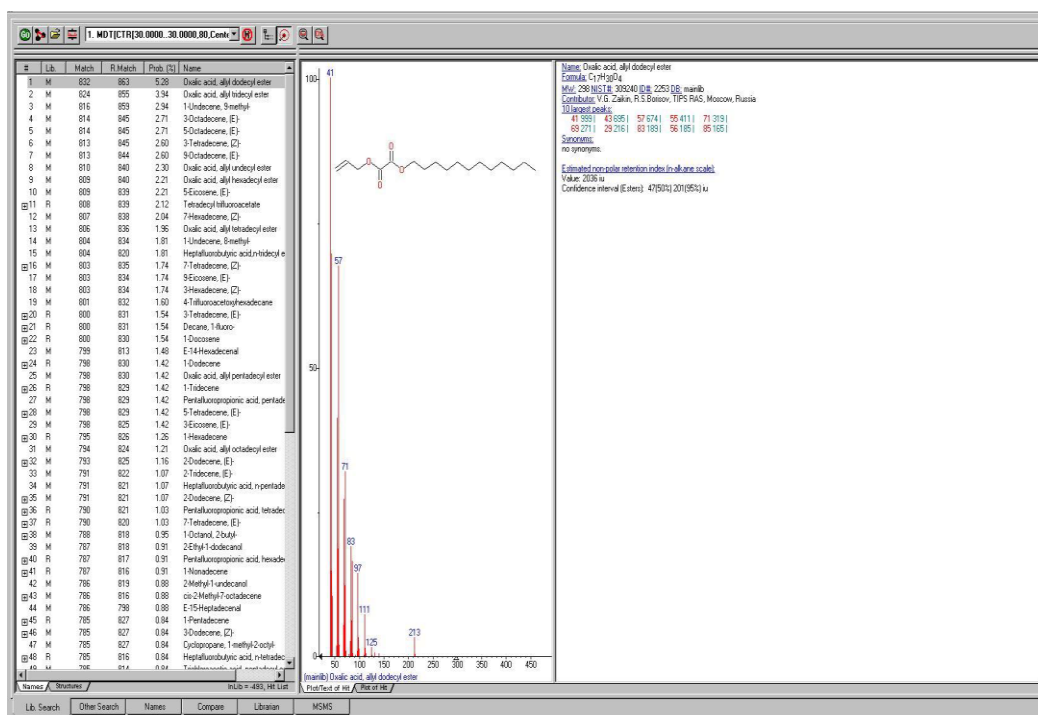


Figure 10: NIST Mass Spectral Library displaying Oxalic acid allyl dodecyl ester as the unknown peak

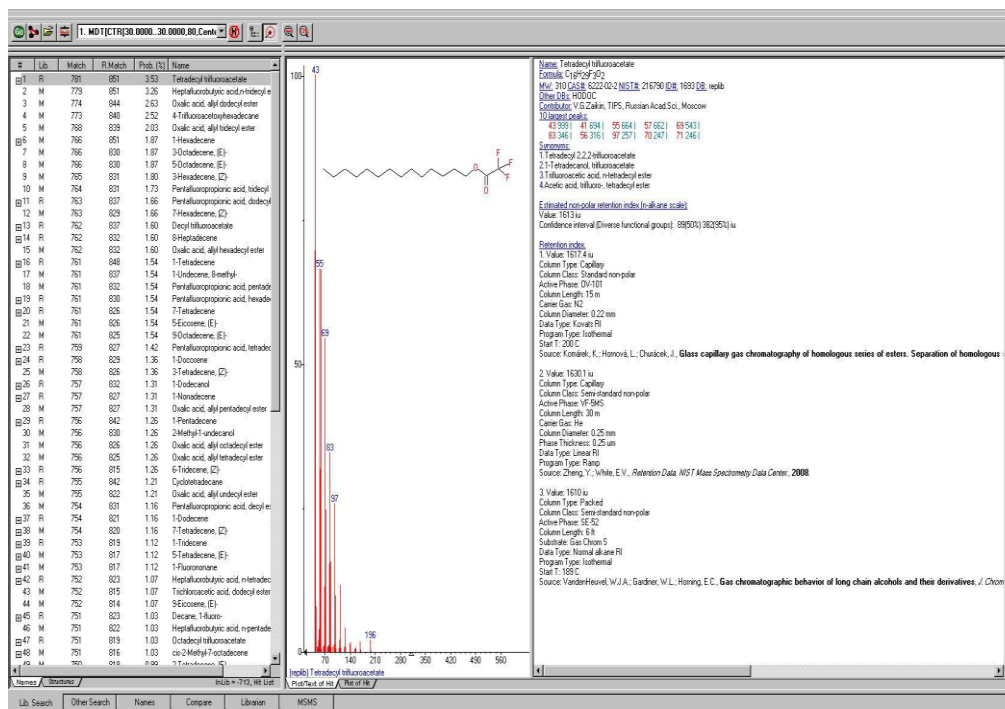
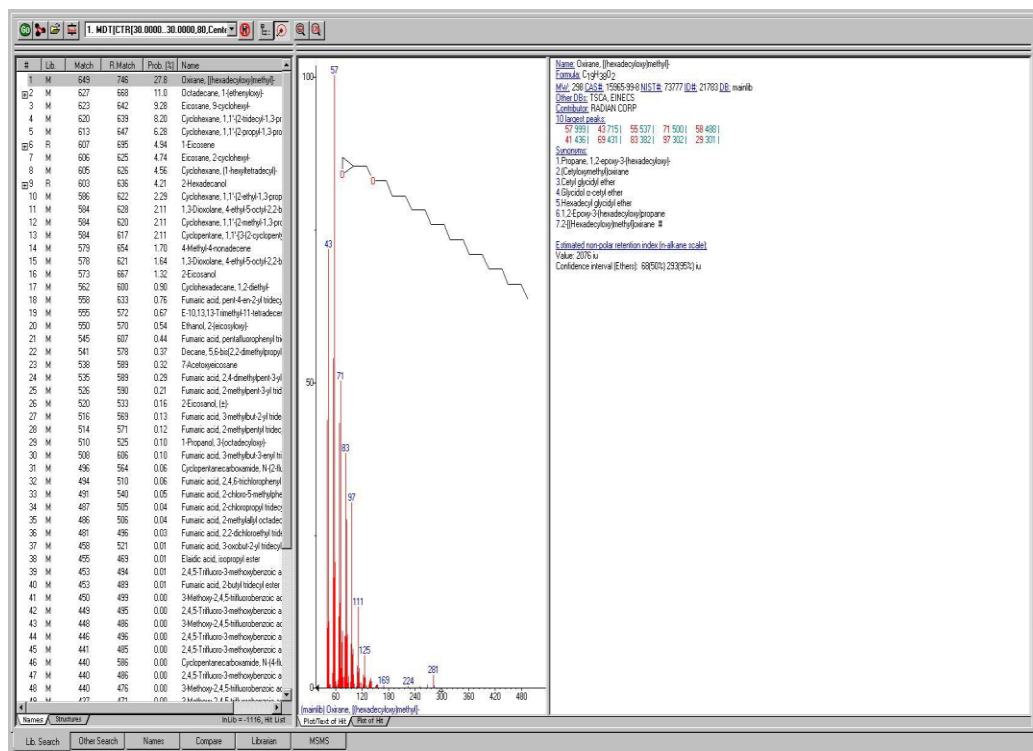


Figure 11: NIST Mass Spectral Library displaying tetradecyl trifluoroacetate as the unknown peak.



7. CONFLICT OF INTEREST

Authors hereby declare that there is no conflict of interest for the research work carried out in this paper.

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Phytochemical Analysis and Antimicrobial Activity of *Cucumis melo var agrestis* (Wild Musk Melon) and *Aegle marmelos* (Bael) Rind Extracts and It's Effect on Seed Germination

S. CHAVAN, P. NAIR, A. GUPTE*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Plants contain wide variety of free radical scavenging molecules like flavonoids, vitamins, phenols that have antioxidant properties. The present study was aimed at evaluating the phytochemical composition, antioxidant and antibacterial activity of *Cucumis melo var agrestis* and *Aegle marmelos* rind extracts and its effect on spinach seed germination. Various phytochemicals were evaluated qualitatively and quantitatively. The total phenolic content observed in the extracts of *C.melo var agrestis* methanolic extract (CMVA-ME), *C.melo var agrestis* aqueous extract (CMVA-AE), *Aegle marmelos* methanolic extract (AM-ME) and *Aegle marmelos* aqueous extract (AM-AE), were 0.005121 ± 0.002 mg of GAE/g, 0.001651 ± 0.0005 mg of GAE/g, 0.4369 ± 0.05 mg of GAE/g, 0.6136 ± 0.37 mg of GAE/g of extract. Determination of flavonoids by aluminium chloride method showed 0.1583 ± 0.02 mg of QE/g in CMVA-ME, 0.0242 ± 0.001 mg of QE/g in CMVA-AE, 0.06 ± 0.02 mg of QE/g in AM-ME and 0.118 ± 0.05 mg of QE/g of AM-AE. Antioxidant activity was found to be $81.02 \pm 0.73\%$ in CMVA-ME, $70.19 \pm 1.29\%$ in CMVA-AE, $95.64 \pm 0.55\%$ in AM-ME and $80.63 \pm 0.34\%$ in AM-AE by 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity. HPLC analysis was performed to determine the phenols present in the extracts. Antibacterial activity of extracts by well-diffusion method indicated that extracts of *C.melo var agrestis* were effective against *Staphylococcus aureus* and *Escherichia coli* while none of the *A. marmelos* extracts showed any activity against the two organisms. A pot experiment was performed to study the effect of rind extracts on seed germination and growth of spinach seedlings.

KEYWORDS: *Cucumis melo var agrestis*, *Aegle marmelos*, Phytochemical analysis, Antibacterial activity, Seed germination, Chlorophyll analysis.

Corresponding Author: Dr. Arpita Gupte

School of Biotechnology and Bioinformatics, Dr. D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: arpita.gupte@dypatil.edu

1. INTRODUCTION

Medicinal plants have been the mainstay of traditional herbal medicine worldwide. The consumption of a plant-based or phytochemical-rich diet has been associated with a reduced risk of chronic human illnesses such as certain types of cancers, inflammation, cardiovascular and neurodegenerative diseases [1]. Phenolic compounds, including flavonoids, anthocyanins and tannins, are the main group of antioxidant phytochemicals that have great value due to their

biological and free radical scavenging activities [2]. The plant parts of *Cucumis melo var agrestis* and *Aegle marmelos* have long been employed for the treatment of various diseases. The fruits of *C.melo* has stomachic properties while fruits of *A. marmelos* have been used as a remedy for diarrhoea. The objective of the present study was to carry out phytochemical analysis of *Cucumis melo var agrestis* and *Aegle marmelos* rind extracts and subsequently to study its antimicrobial activity and its effect on Spinach seed germination.

2. MATERIALS AND METHODS

2.1.Collection of plant material

Fresh fruits of *C.melo var agrestis* and *A. marmelos* were collected from the local fruit vendors at A.P.M.C Market, Navi Mumbai. Spinach seeds were obtained from Konkan Krishi Vikas Pratishthan plant nursery, Kharghar.

2.2.Preparation of extracts

The dried rind pieces were ground to a fine powder. The powder was extracted in a Soxhlet apparatus using methanol and water as solvents. The extracts were concentrated using a rotary evaporator. The residual solvent was further evaporated in the oven to obtain the dried extract.

2.3.Qualitative phytochemical screening

The diluted extracts were evaluated for the presence of phytochemical constituents qualitatively [3-4].

2.4.Parameters related to Antioxidant capacity

2.4.1.Total Phenolic Content (TPC)

Total Phenolic content was determined by the Folin-Ciocalteu (FC) method [5-6]. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE) using the standard curve equation:

$$y = 0.0134x + 0.0158, R^2 = 0.9882$$

where, y is the absorbance at 650 nm and x is the total phenolic content in the extract.

One ml of diluted extracts or standard (20-100 µg/ml) was mixed with 0.5 ml Folin-Ciocalteu (FC) reagent. The mixture was incubated in dark for 3 minutes. Two ml of 20% Na₂CO₃ was then added to the mixture. The mixture was incubated in boiling water bath for 1 minute. The absorbance was measured at 650 nm.

2.4.2.Total Flavonoid Content (TFC)

Total Flavonoid content was determined by the Aluminium Chloride method [7]. Quercetin was used as standard and flavonoid contents were measured as quercetin equivalent (QE) using the standard curve equation:

$$y = 0.3946x + 0.0026, R^2 = 0.9973$$

where, y is absorbance at 510 nm and x is total flavonoid content in the extracts.

To one ml of diluted extracts or standard (2-10 µg/ml), 0.3ml of 5% NaNO₂ was added. After 5 minutes, 0.3 ml of 10%AlCl₃ was added to the mixture. After 1 minute, 2 ml of 1M NaOH was added and volume made up to 10 ml with distilled water. The absorbance was measured at 510 nm.

2.4.3.2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity

The scavenging activity on DPPH radical of rind extract was determined using the method reported by [8]. Ascorbic acid was used as the standard. A diluted rind extract or standard (2- 10 µg/ml) was added to 1 ml of 0.1 mM freshly prepared methanolic solution of DPPH. The mixture was shaken vigorously and left to stand for 30 minutes in the dark at room temperature. The absorbance was measured at 517 nm. The radical scavenging activity was calculated as percentage inhibition by the following formula.

$$\text{Scavenging activity(\%)} = \frac{(\text{A of control} - \text{A of sample})}{(\text{A of control})} \times 100$$

Where A of control is the absorbance of DPPH solution without extract and A of sample is the absorbance of sample with DPPH solution.

2.4.4.Profile of phenolic compounds by High-performance liquid chromatography (HPLC)

Chromatographic separation was achieved on a C-18 reverse phase column (Spherisorb C₁₈, 4.6 x 100 mm, 5 µm particle size). Diluted aqueous extracts (20 µl) were injected and analysed at 280 nm. 20 % methanol in 1 % acetic acid was used as the mobile phase and flow rate of 1.0 ml/min was employed. The diluted extract was identified by comparison of the retention time in chromatogram with standard catechol, caffeic acid, ferulic acid, gallic acid, *p*-coumaric acid and vanillin. Data analysis was done using Empower software.

2.4.5.Screening for antibacterial activity

Anti-bacterial activity was performed using agar well diffusion method [9-10]. The extracts were screened against *Staphylococcus aureus* (ATCC 6538) and *Echerichia coli* (ATCC 2196). The plates were incubated at 37°C for 24 hours. Suitable controls were maintained. After 24 hours, the plates were observed for zone of inhibition. The relative percentage inhibition of the test extracts with respect to positive control was calculated [10-11].

2.4.6.Effect of rind extracts on spinach seed germination and growth

2.4.6.1. Selection of vegetable

Spinach vegetable seeds were selected to study the effect of methanolic and aqueous rind extracts of *C.melo var agrestis* and *A.marmelos* on germination. The seeds were obtained from Konkan Krishi Vikas Pratishthan plant nursery, Kharghar.

The study was carried out as per *Talukder et al.(2015)* [12] with slight modifications. For each treatment,12 seeds of the selected vegetable were washed with distilled water and placed separately in two cups of sizes 6 x 6 cm (6 in each) containing equal amount of potting mix soil. Then 8 ml of each concentration of methanol and aqueous extracts were applied in each pot

respectively while only distilled water was used as the control treatment. 15 ml of distilled water was applied in each cup per day as irrigation. After 15 days, another 8 ml of methanol and aqueous extracts were applied in each respective cups and only distilled water in the control.

The number of germinated seeds were noted daily by visual counting. Germination percentage was calculated as per formula [13].

$$G = \frac{n}{N} \times 100$$

Where, n is the sum of number of seeds germinated and N is the total number of seeds placed for germination.

The chlorophyll a, b and total chlorophyll contents were determined using method described by *Arnon et al. (1949)* according to the method of. Total chlorophyll was determined using the formulae given by *Porra (2002)* [14-15]. Fresh leaves (0.1 g) were macerated using 2ml of 80% acetone and the volume was then made up to 5 ml using 80 % acetone. The mixture was centrifuged at 10,000 rpm for 10 mins at 4°C. The absorbance was measured at 663 nm, 645 nm and 470 nm wavelengths using acetone as reference. The chlorophyll analysis was performed on day 15 and day 30 of seedling growth.

3. RESULTS

The results of preliminary phytochemical screening of methanolic and aqueous rind extracts of *A. marmelos* and *C. melo var agrestis* are summarized in Table 1.

Active constituents	CMVA-ME	CMVA-AE	AM-ME	AM-AE
Tannins	+	-	-	-
Saponins	+	-	-	-
Quinones	++	++	++	++
Flavonoids	++	++	+	++
Alkaloids	+	+	+	+
Glycosides	-	-	-	-
Cardiac glycosides	+	-	-	-
Terpenoids	+	+	+	+
Phenols	+	-	+	-
Steroids	+	+	-	-
Coumarins	+	+	+	++
Anthocyanins	-	-	-	-
Betacyanins	+	+	+	++

Table 1: Phytochemical screening of *C. melo var agrestis* and *Aegle marmelos* rind extracts

(+) Presence of phytoconstituents, (-) Absence of phytoconstituents, CMVA-ME - *Cucumis melo var agrestis* methanolic extract, CMVA-AE - *Cucumis melo var agrestis* aqueous extract, AM-ME - *Aegle marmelos* methanolic extract, AM-AE - *Aegle marmelos* aqueous extract

3.1.TPC, TFC, DDPH radical scavenging activity

Phenolic compounds and flavonoids have redox properties which allow them to act as antioxidants.

Table 2 shows the total phenolic content, total flavonoid content and free radical scavenging activity of the extracts.

3.2.High-performance Liquid Chromatography (HPLC)

The standards used for HPLC were gallic acid, vanillin, catechol, *p*-Coumaric acid, caffeic acid and ferulic acid. Four phenolic compounds viz. gallic acid, catechol, vanillin and *p*-coumaric acid were observed in CMVA-AE and only gallic acid was observed in AM-AE. Table 3 shows that of all the phenolics, Vanillin and Gallic acid were found to be present in higher quantities (Vanillin > Gallic acid > Catechol > *p*-Coumaric acid) Fig.1. Therefore, the antioxidant activity of *C.melo var agrestis* aqueous extract may be majorly due to the presence of vanillin and gallic acid in the extract and that for *A.marmelos* may be because of the presence of gallic acid Fig 2.

Extracts	TPC (mg of GAE/g of extract)	TFC (mg of QE/g of extract)	Antioxidant activity (% scavenging activity)
CMVA-ME	0.005121 ± 0.002	0.1583 ± 0.02	81.02 ± 0.73
CMVA-AE	0.001651 ± 0.0005	0.242 ± 0.001	70.19 ± 1.29
AM-ME	0.4369 ± 0.05	0.06 ± 0.02	95.64 ± 0.55
AM-AE	0.6136 ± 0.37	0.118 ± 0.05	86.63 ± 0.34

Table 2: Total phenolic content, total flavonoid content and free radical scavenging activity of the extracts

CMVA-ME - *Cucumis melo var agrestis* methanolic extract, CMVA-AE - *Cucumis melo var agrestis* aqueous extract, AM-ME - *Aegle marmelos* methanolic extract, AM-AE - *Aegle marmelos* aqueous extract, TPC - Total Phenolic Content, TFC - Total Flavonoid Content, GAE - Gallic Acid Equivalent, QE - Quercetin Equivalent

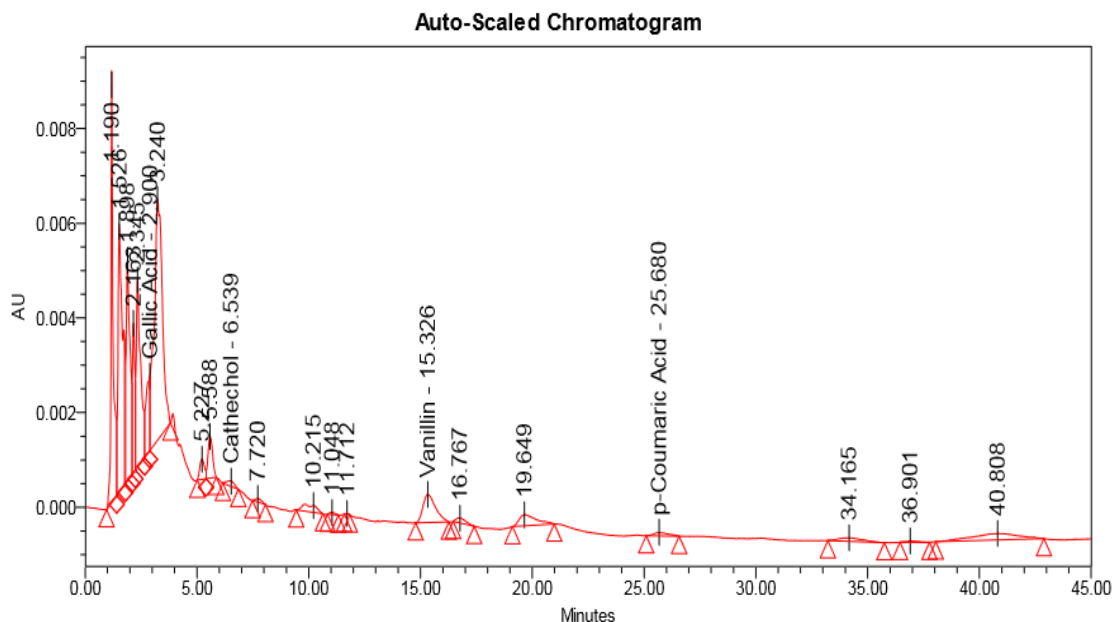


Fig 1: HPLC Chromatogram of polyphenolic profile of CMVA-AE

Phenolic compound	Amount (ppm)	
	CMVA-AE	AM-AE
Gallic acid	0.243	0.231
Catechol	0.095	-
Vanillin	0.261	-
<i>p</i> -Coumaric acid	0.030	-

Table 3: Amount of Phenolic compound present in *C.melo* var *agrestis* and *A.marmelos* aqueous extracts

CMVA-AE - *Cucumis melo* var *agrestis* aqueous extract, AM-AE - *Aegle marmelos* aqueous extract, ppm - parts per million

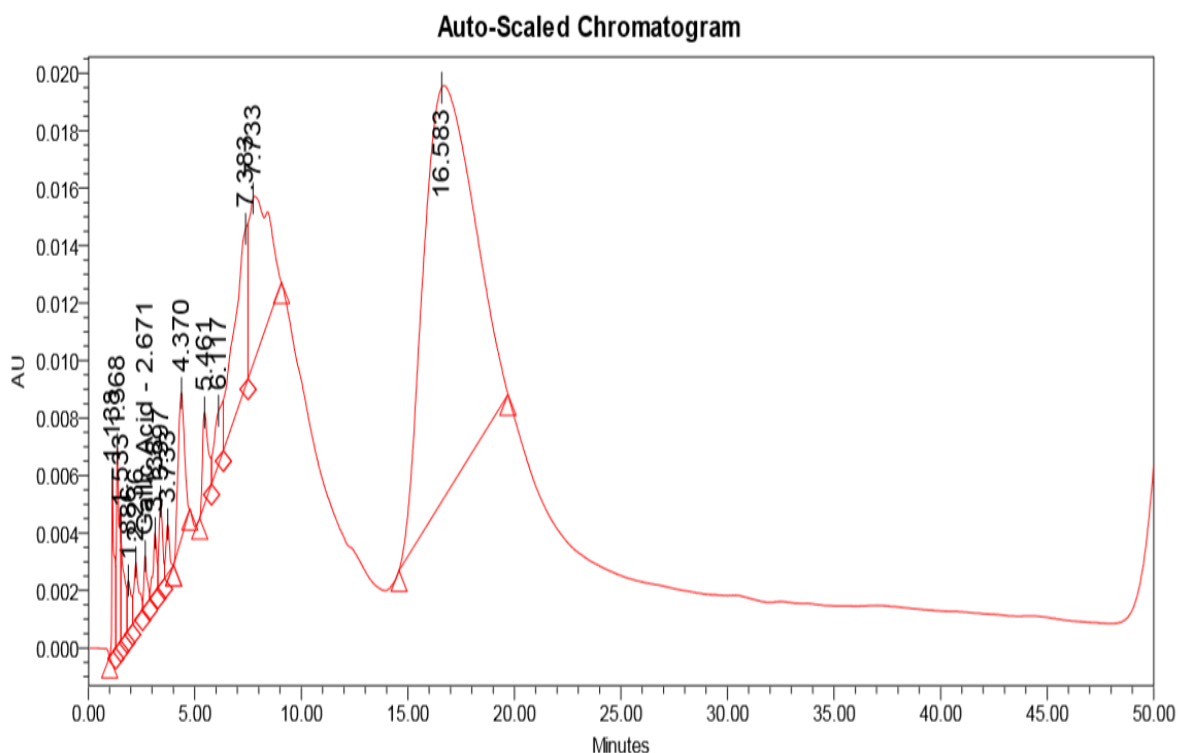


Fig 2: HPLC Chromatogram of polyphenolic profile of AM-AE

3.3.Antibacterial activity

Table 4 represents the zone of inhibition and relative percentage inhibition. *C.melo* var *agrestis* methanolic extract showed greater antibacterial activity against *Staphylococcus aureus* as compared to *Escherichia coli*. Whereas *C. melo* var *agrestis* aqueous rind extract and the extracts of *A.marmelos* showed no antibacterial activity against both the microorganisms.

Extract	Concentration	Zone of inhibition(mm)		Relative percentage inhibition (%)	
		<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>
<i>C.melo var agrestis</i>	100 µg/ml	6.15 ± 0.21	1.9 ± 0.14	3.52 ± 0.19	0.40 ± 0.10
	Crude	8.93 ± 0.11	4.85 ± 0.21	7.42 ± 0.12	2.64 ± 0.23

Table 4: Antibacterial activity of *C. melo var agrestis* methanolic rind extract in terms of zone of inhibition and Relative percentage of inhibition

3.4.Effect of rind extracts on spinach seed germination

Spinach seeds were treated with different concentrations of extracts of *C.melo var agrestis* and *A.marmelos*. Growth was monitored for a period of 30 days, at an interval of 15 days.

The germination percentage of spinach was found to be 16.66% in treatment with 10,000 ppm concentration of CMVA-ME and 5000 and 10,000 ppm concentrations of CMVA-AE on day 1 after sowing, whereas it is least in case of control treatment (Table 5). This suggests that high concentration of CMVA-ME and CMVA-AE promotes seed germination. Also, the germination percentage for each concentration goes on increasing from day 1 to day 7 after sowing. On day 5, 100 ppm and 5000 ppm of CMVA-ME and 5000 ppm and 10,000 ppm of CMVA-AE gives 100% germination percentage test results.

The germination percentage of spinach is highest in control treatment of *A.marmelos* extracts. The extract did not seem to have significant effect in promoting seed germination (Table 6).

Plantlets treated with CMVA-ME showed decreasing growth pattern with increasing concentration of extract over a period of 30 days Fig 3. Plantlets treated with CMVA-AE showed increasing growth pattern with increase in concentration of extract. Slight leaf enlargement was observed in plantlets treated with 1000, 5000 and 10,000 ppm on day 25 Fig 4. Plantlets treated with AM-ME and AM-AE showed decreasing growth pattern. Wilting and drooping of the seedling was observed by day 15, indicating long term contact with the extract will have impact on its growth Fig 5-6.

The chlorophyll content was calculated as per formulae given by Porra (2002) [15]. On day 15 chlorophyll analysis was performed for the seedlings of both the extracts treated with different concentrations. It was found that 500 ppm of *A.marmelos* methanol extract treated plantlets had higher chlorophyll a, chlorophyll b, total carotenoid, and total chlorophyll content than those treated with aqueous extract. While seedlings treated with 5000 ppm of *C.melo var agrestis* aqueous rind extract were found to have better chlorophyll content on day 15. No analysis was performed for extracts of *A.marmelos* on day 30 as all the plants died by day 15 (Table 7, 8).

Conc. (ppm)	Germination percentage (%)											
	1 DAS		2 DAS		3 DAS		4 DAS		5 DAS		7 DAS	
Extracts	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE
Control	0	0	41.66	41.66	75	75	91.66	91.66	100	91.66	100	100
100	0	0	41.66	16.66	75	58.33	100	91.66	100	91.66	100	100
500	8.33	0	33.33	8.33	58.33	41.66	83.33	75	91.66	75	100	91.66
1000	8.33	0	33.33	33.33	58.33	58.33	58.33	91.66	58.33	91.66	75	91.66
5000	8.33	16.66	33.33	41.66	66.66	66.66	91.66	100	100	100	100	100
10,000	16.66	16.66	41.66	16.66	50	50	58.33	100	58.33	100	83.33	100

Table 5: Germination percentage of spinach in the presence of CMVA-ME and CMVA-AE

DAS - Days after sowing, ME - Methanolic extract, AE - Aqueous extract

Conc. (ppm)	Germination percentage (%)											
	1 DAS		5 DAS		7 DAS		10 DAS		12 DAS		15 DAS	
Extracts	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE	ME	AE
Control	0	0	41.6	41.6	75	75	91.6	91.6	100	91.6	100	100
100	0	0	6	6	33.3	8.3	6	6	75	6	83.3	58.3
500	0	0	0	0	3	3	50	33.3	100	58.3	3	3
1000	0	0	0	0	58.3	8.3	91.6	3	58.3	3	100	50
5000	0	0	0	0	3	3	6	25	3	50	58.3	33.3
10,000	0	0	0	0	8.33	8.3	33.3	25	83.3	33.3	3	3
			0	0	0	3	3	83.3	3	3	100	100
					8.33	25	41.6	3	33.3	100	41.6	58.3
						25	6	25	3	58.3	6	3
							33.3	3		3		

Table 6: Germination percentage of spinach in the presence of AM-ME and AM-AE

DAS - Days after sowing, ME - Methanolic extract, AE - Aqueous extract



Figure 3: Growth pattern of spinach seedlings treated with CMVA-ME

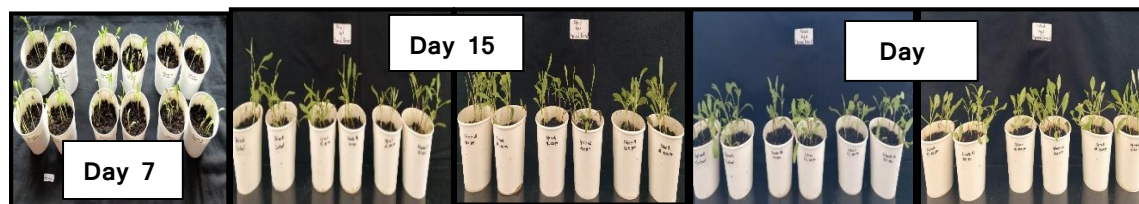


Figure 4: Growth pattern of spinach seedlings treated with CMVA-AE



Figure 5: Growth pattern of spinach seedlings treated with AM-ME

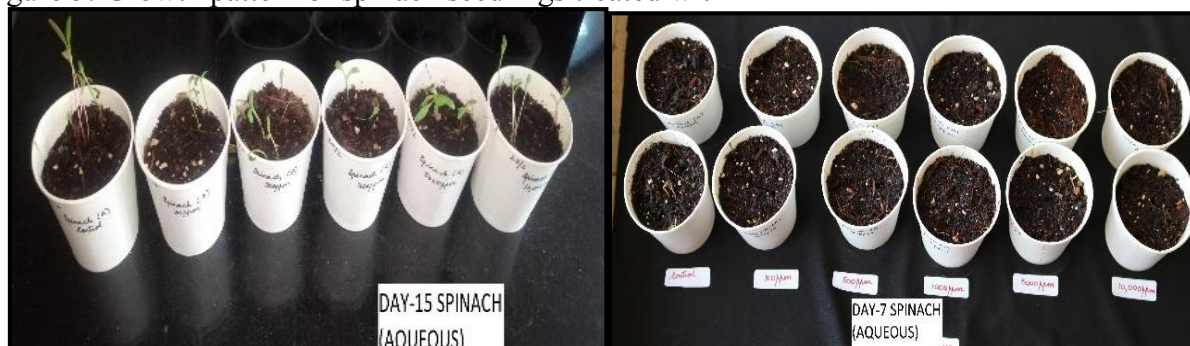


Figure 6: Growth pattern of spinach seedlings treated with AM-AE

Chlorophyll content	Concentration				
Chlorophyll-a	Control	Extract	100ppm	1000ppm	10000ppm
	10.83 ± 0.78	CMVA-ME	12.27 ± 0.74	6.54 ± 0.50	3.30 ± 0.27

		AM-ME	5.24 ± 0.22	4.81 ± 0.16	2.93 ± 0.30
		CMVA-AE	8.17 ± 0.24	11.27 ± 0.35	11.98 ± 0.22
		AM-AE	5.19 ± 0.36	4.11 ± 0.78	2.02 ± 0.46
Chlorophyll-b	2.12 ± 0.47	CMVA-ME	2.15 ± 0.65	1.27 ± 0.37	1.16 ± 0.20
		AM-ME	1.52 ± 0.31	1.53 ± 0.12	0.45 ± 0.08
		CMVA-AE	1.82 ± 0.23	2.87 ± 0.32	2.89 ± 0.31
		AM-AE	1.03 ± 0.24	2.03 ± 0.13	0.37 ± 0.04
Total Chlorophyll	13.48 ± 0.49	CMVA-ME	15.65 ± 0.68	8.16 ± 0.40	5.09 ± 0.63
		AM-ME	6.82 ± 0.15	6.40 ± 0.13	3.42 ± 0.11
		CMVA-AE	1.82 ± 0.57	2.87 ± 0.32	2.89 ± 0.71
		AM-AE	6.28 ± 0.19	6.19 ± 0.39	2.42 ± 0.29

Table 7: Day15 Chlorophyll analysis of *C.melo var agrestis* and *A.marmelos* aqueous and methanolic rind extracts

CMVA-ME - *Cucumis melo var agrestis* methanolic extract, CMVA-AE - *Cucumis melo var agrestis* aqueous extract, AM-ME - *Aegle marmelos* methanolic extract, AM-AE - *Aegle marmelos* aqueous extract

	Concentration	Ctrl	100 ppm	1000 ppm	10,000 ppm
CMVA-ME	Chl-a (mg/g f.wt)	10.83 ± 0.78	12.27 ± 0.74	6.54 ± 0.50	6.30 ± 0.27
	Chl-b (mg/g f.wt)	2.12 ± 0.07	2.15 ± 0.68	1.27 ± 0.37	1.16 ± 0.20
	Total chlorophyll (mg/g f.wt)	13.58 ± 0.47	15.65 ± 0.68	8.16 ± 0.40	7.87 ± 0.28

	Total carotenoid (mg/g f.wt)	2.85 ± 0.76	2.60 ± 0.72	1.80 ± 0.62	2.69 ± 0.22
CMVA-AE	Chl-a (mg/g f.wt)	10.66 ± 0.75	8.17 ± 0.24	11.27 ± 1.35	11.98 ± 1.22
	Chl-b (mg/g f.wt)	1.78 ± 0.50	1.82 ± 0.63	2.89 ± 0.32	2.87 ± 0.71
	Total chlorophyll (mg/g f.wt)	13.48 ± 0.49	11.50 ± 0.71	15.16 ± 0.48	16.83 ± 0.51
	Total carotenoid (mg/g f.wt)	2.54 ± 0.32	3.05 ± 0.17	2.45 ± 0.39	2.86 ± 0.44

Table 8: Chlorophyll analysis day 30

CMVA-ME - *Cucumis melo var agrestis* methanolic extract, CMVA-AE - *Cucumis melo var agrestis* aqueous extract, mg/g - milligram per gram, f.wt - fresh

4. DISCUSSION

The high scavenging property of AM-ME extract may be due to the presence of hydroxyl groups existing in the phenolic and flavonoid compounds chemical configuration that provide the essential constituents as a radical scavenger [16]. Furthermore, significant antioxidant activity in the methanol extract indicated that the selective extraction of antioxidants by appropriate solvent is very important in obtaining fractions with high antioxidant activity [17]. The antibacterial activity of CMVA-ME may be due to the presence of relatively higher content of phenols and flavonoids as compared to its aqueous extract. The medicinally bioactive components exert antimicrobial action through different mechanisms. Terpenoids dissolve the cell wall of microorganism by weakening the membranous tissue. Flavonoids have the ability to complex with extracellular and soluble proteins of bacterial cell wall [18].

The increase in germination percentage of seedlings treated with CMVA-AE may be due to the presence of vanillin, as its concentration is indicated by the HPLC profile. The increase in the growth pattern of plantlets treated with CMVA-AE may be due to the presence of polyphenols in extracts that seem to increase IAA-mediated growth by inhibiting IAA oxidative decarboxylation [19]. Studies have also shown that gallic acid inhibited plant growth regardless of their concentration [13]. Thus, the decrease in the growth pattern of plantlets treated with the AM-AE may be due to the presence of gallic acid as indicated by the HPLC profile.

5. CONCLUSION

The results obtained in this study indicate the antibacterial activity of methanolic rind extract of *C. melo var agrestis* was more effective against *S. aureus* than against *E. coli* as indicated by relative percentage of inhibition. Whereas, its aqueous extract and extracts of *A. marmelos* showed no inhibitory activity. The low inhibitory activity may be due to relatively low content of phenols and flavonoids in the extracts. HPLC analysis of CMVA-AE showed the presence of highest amount of vanillin followed by gallic acid. Both the extracts of *C. melo var agrestis* seemed to promote germination but only aqueous extract have showed to enhance the growth pattern of spinach as opposed to methanolic extract. This suggests that CMVA-AE could be used as a possible plant growth enhancer or as a biofertilizer. The presence of phytochemical compounds in the extracts including polyphenols like vanillin, catechol and flavonoids as major active constituents may be responsible for those activities. The high antioxidant property of *A. marmelos* extracts could be used as additives and substitutes for synthetic compounds in foods. Thus, in the near future rind extracts of *Cucumis melo var agrestis* and *Aegle marmelos* could be a possible source of useful phytochemical compounds that may play a vital role in modern medicine, food and agriculture.

6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

7. ACKNOWLEDGEMENT

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Mapping of Antibiotic Resistant Bacteria from Coastal Water Bodies of Mumbai and Suburban Areas

A SALUNKE, S BHOWMIK, N MUKHERJEE, AND S GHOSH*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Prevalence of antibiotic resistant microbes in nature is considered to be a global threat for human population. Biotic and abiotic zones of highly populated cities are considered to be the incubator zones of such microbes all over the world. To review the above concern, we aimed to map the antibiotic sensitivity profiles of cultivable bacteria, in surface water bodies of coastal Mumbai and suburban areas. Thus to identify the distribution pattern of possible polluted sources was the prime focus of the study. Standard two step culture dependent technique was used to evaluate the antibiotic resistance of bacterial population. Thirty water samples were collected from all around Mumbai and the sensitivity profile were checked with ten standard antibiotics belonging to 1st, 2nd, 3rd and 4th generation. Mixed antibiotic sensitivity was noted among the 310 (31x10) observations. Regardless of the geographical positions, all isolated bacterial population were sensitive to Tigecyclines, Ceftriaxone & Doxycyclines. However, bacterial population from 28 out of 30 tested samples showed resistance to Trimethoprim and Colistin. The most polluted sites with regards to antibiotic resistant bacteria were Trombay, Girgaon, Bhyandar East and Dharavi fort. The bacterial population from the above samples were found to be resistant against 9 out of 10 tested antibiotics. The cultivated bacterial populations from samples harbor resistance upto 3rd generation antibiotic. Although the antibiotic sensitivity map was not linked with the distance of the selected sampling points but the inclusive presence of 4th generation colistin resistant bacteria would definitely be an annoyance for public health. Overall the study represents the insight of distribution pattern of antibiotic resistant organisms in the surface water system of Mumbai and suburban areas for which monitoring system is utmost important to address.

KEYWORDS: Antibiotic, resistance, water, coastal region

Corresponding Author: Dr. Sourav Ghosh

School of Biotechnology and Bioinformatics, Dr. D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: Sghosh69@gmail.com

1. INTRODUCTION

Antibiotic resistance (AR) is considered as a global burden in 21st century. Since the discovery of first antibiotic, Penicillin in 1928, by Scottish scientist Alexander Flemming, the threat of antibiotic resistance persist [1]. Aberrant use of antibiotics creates selective pressure, allowing bacteria to develop necessary mechanisms for multiple antibiotic resistances. Thus, antibiotics become no longer the last line of defence system to combat with microbes. Till last decade the studies focused on monitoring antibiotic resistance in hospitals and other clinical environments,

but the rise of community acquired infections of resistant bacteria has driven interest in AR genes in natural environments [2]. Recently several studies indicated that the natural environments including soil [3], glacier [4], water bodies [5], and animals [6] can act as reservoir for antibiotic resistant bacteria. Marine water bodies are mainly dilute environment, thus production of antibiotic resistance gene can be promptly dispersed & exposed to a large amount viable ecosystem in the water bodies.

Three possible ways were proposed by the researcher by which antibiotic resistance can be disseminated through the marine environment. First, by means of coastal region runoff where the non- native antibiotic resistant bacteria of terrestrial sources or commercial antibiotics were thought to mix with marine environment. The second mechanism might be the overuse of antibiotics in marine animals like fishes for either prophylactic or therapeutic purposes. The third mechanism was the production of antibiotics by marine organisms which eventually create a selection pressure to the microbes and subsequent production of resistance [7, 8, 9].

The runoff through coastlines is considered as the most prevalent sources of antibiotic resistance in marine environment. Extensive number of studies on coastal region depict the fact that the coastal regions are facing enormous (uncontrolled) stress though terrestrial or domestic waste which ultimately lead to the introduction of enteric drug resistant pathogen in the marine environment. Considering the Indian perspective, very few studies have shown the presence of drug resistant bacterial strains of *Pseudomonas*, *Staphylococcus* at the costal water bodies [10, 11].

Mumbai is one of the main coastal stations on the west coast of India. It is surrounded with different creeks expanded though 71km² whereas the total accessible coastal area is about 167 kilometre stretched. The Government of India's surveillance program "Coastal ocean monitoring and prediction system" (COMAPS) designated Mumbai coastal area as "hot spot" [12]. With population density of 73,000/mile² it is considered as the second most densely populated cities in the world [13]. Several reports are documented on the prevalence of antibiotic resistant microbes throughout the terrestrial region of the Mumbai and suburban areas but a very few studies have encompassed the antibiotic resistance profile of the coastlines of Mumbai [14]. The coastal regions of Mumbai receive different industrial wastes as well as treated and untreated sewages from manual origin. Apart from that the sewage from the hospitals and recreational origins are also believed to mix with the coastal water [14]. The ever-growing urbanization also leads to the picture more severely than ever in coastal ecosystem of Mumbai. A significant number of native population of Mumbai is dependent upon the fish farming for generations and the economy is directly dependent on it [15]. Recent reports on overuse of antibiotics for fish farming, is believed to have bio magnification effect on ecological pyramid [16]. Hence it can be hypothesised that the antibiotic resistance might also be dispersed in the coastal water similar to terrestrial origin. Therefore the surveillance on coastal water is of utmost importance.

Here with the above goal, we tried to map the antibiotic resistance profile throughout Mumbai as well as its suburban coastal areas. Thirty one (31) coastal regions have been sampled and ten (10) antibiotics of different classes' ranging from 1st generation to 4th generation were taken for resistance pattern study.

2. MATERIAL & METHODS

2.1.Site selection

Sub surface samples from the coastal water were collected from thirty one different sites dispersed from north to south as well as east to west side of Mumbai and suburban areas. A total of 4355km² area has been covered in the present study. All the water samples were collected in sterile PET bottles maintaining the sterile procedures. The hydrographic parameters like pH and Temperature were recorded at the time of collection and the details of the parameters along with site description has been documented in Table 1. The locations of sample collection are shown in Fig 1.

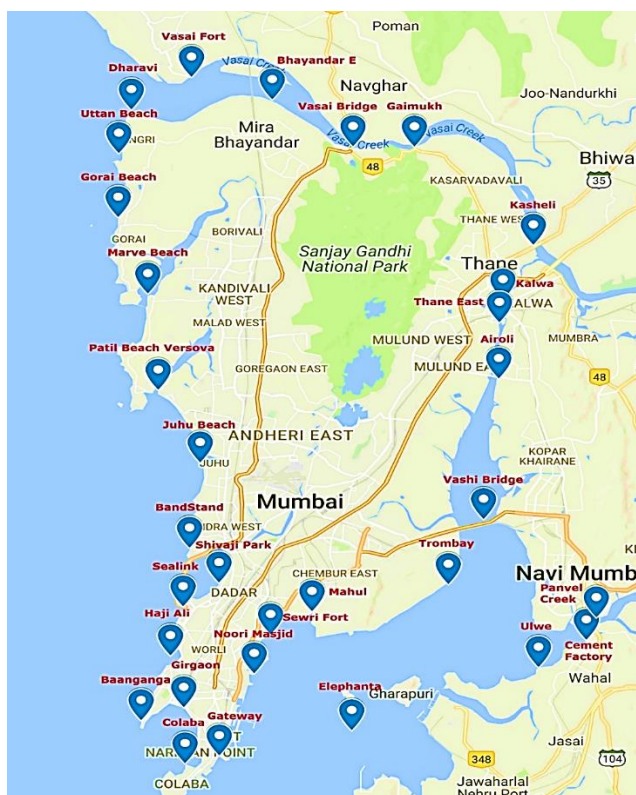


Figure 1: Location of the Sampling sites

2.2.Antibiotic sensitivity assay

The antibiotic sensitivity test was adopted from Mishra *et al* 2012 with minor modification [17]. Wells of polystyrene plates (Tarson Cat. No. 980030)) were filled up with one ml of sterile total plate count liquid medium. Next, pre-coated sterile antibiotic discs (HiMedia) were dipped into each media containing wells and put the plates in 4°C for 30 minutes for proper diffusion. 50µl of sub surface water samples were directly given to each antibiotic containing well and incubated overnight at 25°C for growth. Two wells were left as positive (no antibiotic disc) and negative control (no water sample). All the experimental sets were replicated at least thrice and the resistant mixed bacterial population were preserved with 70% glycerol at -80 °C throughout the study.

3. RESULTS & DISCUSSION

3.1.Site selection

The sites have been selected with three criterion mind: Presence of industry, Tourist spots and lastly Urban or Rural location. Our observation revealed the pH range was weak acidic to neutral throughout the coastal regions in both sides (Table 1). Usually, the pH fluctuation mostly observed in different industrial sites, due to mixing of acidic to alkaline wastes, although the dilution factor of streamline creeks and coast lines could nullify the effect of the above chemicals. Therefore here we observed a neutral pH range in the study. The salinity depicts the concentration of mineral salts into the marine water bodies. Here we found an average of 28.5 ppt is lower than 37 ppt as reported earlier [18]. The sub surface water depth (5 to 10 meters) as well as the temperature might be the factors for it. The density of the population is taken from the census report of Mumbai and suburban areas. Here we also illustrated the fishing sites which were thought to be significant source of antibiotic resistance.

3.2.Antibiotic sensitivity

The antibiotic sensitivity assay was simplified form of Kirby Bauer method with the detection of resistant and sensitive organism by unaided eyes. Although, by the present method we cannot determine the “intermediate” response as such but the resistant and sensitivity profile could easily be determined by comparing the growth ($OD_{600} \sim 0.45$) of the microbes in the presence and absence of antibiotic in the culture media. Overall the present methods could aid in antibiotic resistance profile directly from the environmental samples (here marine water) within less than 24 hours. The optimum growth period of most of the pathogenic organism can be observed within 24 hours, thus the above experimental condition could effectively elucidate the antibiotic resistance profile of the isolated pathogenic microbes from marine origin. The details of 10 antibiotics used here were given in Table 2.

The antibiotic resistance profile was found to be of “mixed” nature depicted in Table 3. Figure 2 displays the distribution pattern of resistant microbes for various antibiotics used in the study. The colours represent the category of antibiotic such as: Yellow for 1st generation, Orange for 2nd generation and Red for 3rd generation. Each pie chart depicts the proportion (in percentage) of antibiotics to which the microbes from the water sample showed growth (resistant microbes). None of the culture depended bacterial population from the water samples were found resistant to 4th generation glycyclines antibiotic, Tigecyclines. Glycyclines group of antibiotics are considered to be the most effective in this era while a few number of study according to our understanding could report the resistant microbes towards the same drug [29]. The antibiotic resistance profile exhibited by water samples of various sites could not be corroborated with their geographical closeness. In case we found the post polluted places are Bhyandar, Dharavi fort and Colaba in west coast whereas Airoli and Trombay in east coast were the most polluted sites in terms of number of antibiotics to which samples showed resistance among the tested antibiotics. The common factor of the above stated places is the inhabited population density. Thus the sewage containing antibiotic resistant bacteria from manual excreta as well as huge load of house hold waste may impart resistance among the coastal marine micro flora. Surprisingly there were very few 3rd

generation antibiotic resistant microbes found in tourist spots although the presence of 1st and 2nd generation antibiotic resistant was evident in these places. Another noteworthy feature is the establishment of different industries in either sides of Mumbai. The untreated effluent from Pesticide, Pharmaceuticals and petrochemical industries might have a significant impact on antibiotic resistance [30]. These coastal regions of Mumbai are also affected by the huge fishing activity [15]. Therefore the resistant bacteria from fishermen might also have a role to play. The antibiotics like colistin or Polymixin E were considered “drug of last resort” against pathogenic gram negative bacteria [31].

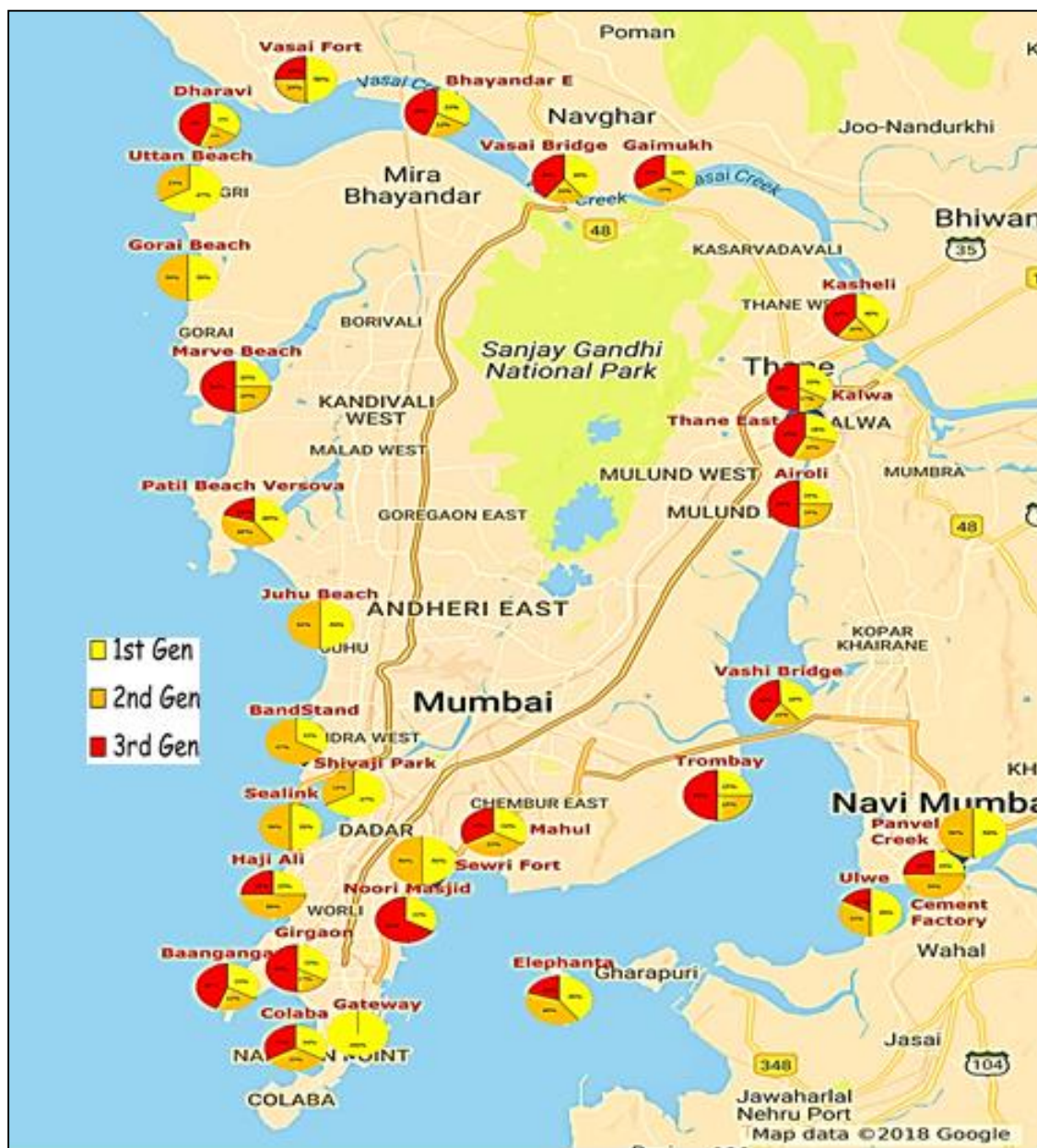


Figure 2: Details of Antibiotic resistant Profile of the cultivable bacteria from different water samples

Our investigation exhibited presence of colistin resistant organisms in 28 out of 30 tested water samples. Although our further investigation showed mixed bacterial population in those samples but the cross resistance pattern against polymyxins were also evident [32]. In the year 2017, Islam & coworkers [33] found colistin resistant strains of *E.coli* from sludge samples. Thus it can be anticipated that the untreated samples from municipal and industrial sludge might play a pivotal role to impart colistin resistance in marine samples. Similar finding has also found in case of sulphonamide drug Trimethoprim. Most of the Tuberculosis bacillus patients usually are given the drug for treatment [34], the presence of resistant organism might also highlight the presence of drug resistant Mycobacterium in the marine water samples. There is very weak or no correlation found between the geographical location and the antibiotic resistance profile. The tidal effect and the rains would be justifiable reason for it. The mixed pattern of antibiotic resistance could also point out the dispersion of antibiotic resistance throughout the coastal water bodies of Mumbai and suburban area, which definitely raises a concern regarding the healthcare of inhabitants exposed to it.

Sampling Sites	Co-ordinate	pH	Temperature (°C)	Salinity (ppt)	Nature of the sites
East Coast					
Vasai bridge	19.287304, 72.905765	6.7	24.5	23.4	Rural
Gaimukh	19.287250, 72.938472	6.3	24.1	20.4	Rural/Fishing Area
Kasheli	19.228495, 73.001569	6.5	23.8	18.1	Rural
Kalwa	19.195861, 72.985472	7.5	23.5	18.2	Populated
Thane E	19.182139, 72.983500	7.5	24	15.1	Urban
Airoli	19.148583, 72.983245	6.7	23.6	22	Urban
Vashi	19.063389, 72.975194	6.3	24.5	32.7	Urban
Trombay	19.025482, 72.956388	7.5	25	30.8	Industrial
Panvel Creek (high)	19.005417, 73.035111	6	23.5	29.9	Industrial
Mahul (No)	19.009182, 72.884063	7.6	23	7.4	Industrial
Sewri fort	18.995333, 72.862083	7.5	24.2	34.1	Industrial spot
Noori Masjid	18.972090, 72.853345	6.5	23.5	32.7	Industrial spot
Elephanta	18.937972, 72.905028	6.5	24.8	34.2	Tourist Spot
Gateway	18.922928, 72.834670	6.5	24.4	31.2	Tourist Spot

Ulwe	18.975833, 73.004278	6.3	24.3	33.7	Rural
Cement Factory	18.992250, 73.029663	6	24.5	30.2	Industrial
West Cost					
Bhyandar E	19.317778, 72.859556	8	26.5	21.1	Populated
Vasai Fort	19.328764, 72.819983	6.5	25	27.8	Rural
Dharavi Fort	19.309222, 72.787806	7	25	31.8	Populated
Uttan Beach	19.283210, 72.781259	6	24.5	32.6	Tourist Spot
Gorai Beach	19.245000, 72.780917	6.5	23	32.1	Tourist Spot
Marve Beach	19.199349, 72.796764	7	23	32	Tourist Spot/Rural
Patil Beach	19.141750, 72.802361	6.5	24.3	35	Tourist Spot/Rural/Fishing area
Juhu Beach	19.098355, 72.824578	6	25	33.1	Tourist Spot/Populated
Band Stand	19.047139, 72.818861	6.5	24.4	34.1	Tourist Spot/Urban
Shivaji Park	19.026396, 72.834530	7.5	22.5	32.2	Tourist Spot/Populated
Sealink	19.012194, 72.815500	6.5	23	33.2	Tourist Spot
Haji Ali	18.986617, 72.808296	7.3	22	33.8	Tourist Spot/Urban
Baanganga	18.944028, 72.793250	6.5	23.7	24.7	Tourist Spot/Rural
Girgaon	18.951692, 72.815884	6.7	24	35.7	Tourist Spot/Urban
Colaba	18.918167, 72.816500	7	23.6	31.2	Urban/Populated/Fishing Area

Table 1: Details of Sampling Sites

4. CONCLUSION

Our study could holistically represent the map of antibiotic resistance of the coastal regions of Mumbai and its suburban areas. The detailing of the study is first of its kind according to our knowledge for Mumbai coastal region. The observed results raise concern over such wide antibiotic resistance pattern that needs to be curbed for overall health of the marine ecosystem and human population. More detailed study with farther extension may provide clearer picture of the existence of such resistance pattern in the marine ecosystem.

Antibiotics	Class	Mode of Action	Spectrum	Reference
1st Generation Antibiotic				
Trimethoprim	Sulphonamide	DNA synthesis blocker	Broad Spectrum ^{#*}	19
Doxycycline	Tetracycline	Protein synthesis inhibitors	Broad Spectrum ^{#*}	20
Streptomycin	Aminoglycoside	Protein synthesis inhibitor	Broad Spectrum ^{#*}	21
2nd Generation Antibiotics				
Norfloxacin	Fluoroquinolone	DNA synthesis blocker	Broad Spectrum ^{#*}	22
Colistin	Polymyxin	Membrane Integrity Inhibitor	Narrow spectrum [*]	23
3rd Generation Antibiotics				
Sparfloxacin	Quinolone	DNA replication and transcription inhibitor	Broad Spectrum ^{#*}	24
Vancomycin	Glycopeptide	Cell wall synthesis inhibitor.	Narrow spectrum [#]	25
Azithromycin	Macrolide	Protein synthesis inhibitor.	Broad Spectrum ^{#*}	26
Ceftriaxone	Beta-lactum	Cell wall synthesis	Narrow spectrum [#]	27
4th Generation Antibiotic				
Tigecycline	Glycylcycline	Protein synthesis inhibitor	Broad Spectrum ^{#*}	28

Table 2: Details of Antibiotics used in the study

Sampl ing Sites	Tigecy cline (TGC)	Norfloxa cin (NX)	Colisti n (CL)	Trimetho prim (TR)	Doxycycli ne (DO)	Sparflo xacin (SPX)	Streptomy cin (S)	Vancom ycin (VA)	Azithrom ycin (AZN)	Ceftri axone (CTR)
West Coast										
Vasai Fort	S	S	R	R	S	S	R	S	R	S

Uttan Beach	S	S	R	R	S	S	R	S	S	S
Marve Beach	S	R	S	R	S	S	S	R	R	S
Juhu Beach	S	S	R	R	S	S	S	S	S	S
Shivaji Park	S	R	S	R	S	S	R	S	S	S
Haji Ali	S	R	R	R	S	S	S	S	R	S
Girgaon	S	S	R	R	S	S	R	R	R	R
Bhandardar	S	R	R	R	R	R	R	R	R	R
East Dharvi fort	S	R	R	R	R	R	R	R	R	R
Gorai beach	S	R	R	R	S	S	R	S	S	S
Patil Beach	S	R	R	R	S	S	R	S	R	S
Band Stand	S	R	R	R	S	S	S	S	S	S
Sealink	S	S	R	R	S	S	S	S	S	S
Baanga	S	R	R	R	R	R	R	R	R	R
Colaba	S	R	R	R	S	S	R	R	R	S
East Cost										
Sampl es	Tigecy cline (TGC)	Norfloxa cin (NX)	Colisti n (CL)	Trimetho prim (TR)	Doxycycli ne (DO)	Sparflo xacin (SPX)	Streptomy cin (S)	Vancom ycin (VA)	Azithrom ycin (AZN)	Ceftri axone (CTR)
Vasai Bridge	S	S	R	R	S	S	R	R	R	S
Kasheli	S	S	R	R	S	S	R	R	R	S
Airoli	S	R	R	R	S	R	R	R	R	R
Mahul (N)	S	R	S	R	S	S	S	S	R	S
Trombay	S	R	R	R	S	R	R	R	R	R
Noori Masjid	S	S	S	R	S	S	S	R	R	S

Cement Factor y Gateway Gaimukh point Kalwa bridge Thane East Vashi bridge Panvel creek Ulwe Sewri fort Elephanta	S	R	R	R	S	S	S	S	R	S
	S	S	S	R	S	S	S	S	S	S
	S	R	R	R	S	S	R	R	R	S
	S	S	R	R	S	S	R	R	R	R
	S	R	R	R	S	R	R	R	R	S
	S	S	R	S	S	S	S	S	S	S
	S	S	R	R	S	S	S	S	S	S
	S	R	R	R	R	S	R	S	R	S
	S	R	R	R	S	S	R	S	S	S
	S	R	R	R	S	S	R	R	S	S

Table 3: Details of Antibiotic resistance profile of respective sampling sites

R= Resistant; S=Sensitive

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6. CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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Molecular Marker Based Assessment of Genetic Fidelity of Tissue Cultures of *Barleria prionitis* L.

N. JOSHI, A. PANCHAL, S. SINGH*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50, Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: *Barleria prionitis* L. is a well explored Indian medicinal plant valued for its stem and leaf which forms an important ingredient of many ayurvedic formulations. It is used for the treatment of various disorders like toothache, bleeding gums, strengthening gums, whooping cough, inflammation, arthritis, enlargement of scrotum and sciatica etc. The plant is propagated vegetatively through stem cuttings. Poor seed viability and frequent harvesting of planting material drive the need for conservation of this plant. Plant tissue culture technology offers a very good alternative for rapid propagation plant species. However, the plant need to be assessed for its true-to-type nature. For this purpose molecular markers were employed in the present study. DNA extracted from mother plant, callus and *in-vitro* raised plants were amplified using six ISSR and RAPD primers. Callus and plants subjected to random amplified polymorphic DNA and inter simple sequence repeat (ISSR) marker based profiling revealed a uniform banding pattern identical with that of donor plants. Dendrogram constructed using SPSS showed around 75 % genetic similarity between micropropagated and mother plants. The result of the present study warrants the application of tissue culture technology for germplasm conservation of *B. prionitis*

KEYWORDS: callus, dendrogram, molecular markers, somoclonal variation, ISSR, micropropagation,

*Corresponding Author: Dr. Sunita Singh

School of Biotechnology and Bioinformatics, Dr. D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: sunita.singh@dypatil.edu

1. INTRODUCTION

The genus *Barleria* belongs to the family *Acanthaceae* and is comprised of 230 species of herbs and shrubs distributed chiefly in the tropical and subtropical parts of the world. About 30 species occur in India, many of which are known for their ornamental and/or medicinal value. Some of the important species of this genus are *B. prionitis*, *B. greenii*, *B. albostellata*, *B. cristata*, *B. gibsoni*, *B. strigosa*, *B. tomentosa* etc. [1]. *B. prionitis* L. also known as Porcupine plant is an erect, prickly shrub, usually single-stemmed, growing to about 1.5 m tall. The whole plant or its parts like leaf, root, stem, bark and flower has been widely utilized for traditional Indian medicine to cure catarrhal infections, swellings, whooping cough, inflammations, glandular swellings, UTI, fever, gastrointestinal infections and diuretic. It is also known as vajradanti as it makes the teeth strong and free from all diseases. Its antiseptic properties have been reported and the extracts are incorporated into herbal cosmetics and hair products to promote skin and scalp health [2]. Report

suggests that whole-plant extracts of this porcupine shrub contains a wide range of bioactive compounds that exerts both biological and medicinal properties [2].

Over-utilization of medicinal plants have affected their supply and resulted in loss of genetic diversity. *Barleria prionitis* L. is listed under vulnerable species of Central Eco- Region in the Madhya Pradesh Biodiversity Board Survey (RLHT, 2006) [3]. Hence there is a need for conservation of this medicinal plant. The tissue culture technology has been envisaged as an alternative means of germplasm conservation to ensure the survival of endangered plant species and their rapid mass propagation. The tissue culture aims for obtaining true-to-type plants to maintain the germplasm however, exposure to sterilants, plant growth regulators and culture conditions may cause genetic variation among the regenerants which may lead to genetic aberration (methylated DNA, chromosome rearrangements and point mutations) commonly referred as 'somaclonal variations' [4]. Although somaclonal variations may be used as a source of variation to get superior clones, it could be a serious problem in plant tissue culture industry where the aim is to develop identical propagules of a desired variety. With the intention of germplasm conservation, incidence of somaclonal variation limits micropropagation protocol. This necessitates a stringent quality check of the plantlets obtained from micropropagation to screen for its clonal fidelity [4].

The traditional methods viz. morphological, cytological and biochemical markers have been currently complemented / replaced by the fast and reliable molecular markers to detect and monitor the genetic fidelity of tissue culture derived plantlets. This is apparent in studies conducted to screen somaclonal variations produced in tissue culture derived plants like bamboo [5], *Paederia foetida* L. [6], banana [7]. Establishing the genetic identity of these regenerants makes the use of molecular markers an attractive tool for identification. Past few years have witnessed the application of different types of markers for these purposes.

Applying DNA based PCR molecular markers for assessment of genetic variation in plants has shown advantages over other markers based on hybridization and the phenotype; the former being neutral, not related to age and tissue type, not influenced by the environmental conditions, feasibility, lower costs and more informative than morphological markers. Among the PCR based reliable DNA markers Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are two of the most popular markers that have been widely used in population genetic studies to characterize genetic divergence within and among the populations or species of various medicinal plants [8, 9, 10].

The selection of RAPD and ISSR is based on their relative technical simplicity, level of polymorphism they detect, cost effectiveness, easily applicable to any plant species and target those sequences which are abundant throughout the eukaryotic genome. Recent studies on *Barleria* spp [11] have also used barcode genes like *rbcL*, *matK* gene sequences along with RAPD and microsatellite based markers [12] to determine the genetic variation. While very few scientists have attempted the tissue culture of this plant, [3, 13] none of them have tested their genetic fidelity. To our knowledge, no report was available on the comparative genetic stability of regenerants and mother plant of *B. prionitis* by using RAPD and ISSR markers. This research is first such study related to genetic fidelity testing of *B. prionitis* L. obtained by callus organogenesis thus laying a stepping stone and a wide area of research in molecular study open to all scientists considering the

medicinal importance of this plant.

2. MATERIALS AND METHOD

2.1.Plant material

For tissue culture and genetic fidelity studies the plants of *Barleria prionitis* L. were collected from Thane, Maharashtra. The plant material was identified and authenticated at Botanical survey of India, Regional Research Centre, Pune. For callus induction the leaves were carefully removed from healthy, disease free plants of *B. prionitis* L. maintained in the green house at the School of Biotechnology and Bioinformatics. The explants were then carefully washed under running tap water, surface sterilized with sodium hypochlorite (1% active chlorine) for 7min, followed by wash (3X) with sterile distilled water. The explants were surface sterilized under aseptic conditions by treating with 0.1% HgCl₂ for 8-10 min. This was followed by three times wash with sterile distilled water. The leaves were cut into small pieces and then inoculated onto MS media supplemented with 1.0 mg/l IAA+ 0.5 mg/l BA + 0.03% ascorbic acid. For organogenesis, six month old callus was inoculated on MS media supplemented with 1.0 mg/l IAA + 0.5 mg/l BA + 0.05% activated charcoal. All the cultures were incubated at 25 ± 2° C under 16 hours of photoperiod provided by cool white fluorescent lights in the growth room.

For genetic fidelity studies following materials were used: Field grown mother plant (1), Mother plant leaf-derived callus of various age viz. 1 month old (2), 2 month old (3), 3 month old (4), 4 month old (5) and 5 month old (6), callus-derived cultures from different stages of micropropagation viz. one month old shoot culture (7), multiplication phase shoot culture (8), rooted shoot (9) and hardened plantlet (10).

2.2.DNA extraction from leaves, tissue cultured shoots and callus of *Barleria prionitis* L.

50mg of each sample was crushed with liquid nitrogen and was transferred into sterile 2ml microcentrifuge tube containing 1ml CTAB extraction buffer and 1µl β mercaptoethanol. The tubes were placed in 65°C water bath for 30 min followed by centrifugation at 8000rpm at 4° C for 15min. To the supernatant equal volume of chloroform: IAA (24:1) was added, centrifuged at 6000rpm at room temp for 15min. The supernatant was mixed with ½ volume of 5M NaCl and 1/10th volume of 3M Na-acetate and 2 volume of chilled absolute ethanol and incubated at -20°C for 30min followed by centrifugation at 3000rpm at 4°C for 5 min. The pellet was washed twice with 70% ethanol and resuspended in 50µl 1x TE buffer following centrifugation at 3000rpm at 4°C for 5min to remove any leftover debris. 0.5µl RNase A was added to the supernatant and incubated at 37°C for 30min. Sterile distilled water was added to it to make up volume to 500µl. The DNA was precipitated as mentioned in the earlier step and suspended in 50µl of 0.1x TE buffer and stored until used. The DNA purity was checked at 260 and 280nm and the ratio of 260:280 were calculated.

2.3.RAPD and ISSR analyses

A total of 20 primers (15- RAPD and 5- ISSR) were screened out of which six RAPD and five ISSR primers were selected for analysis (Table 1 and 2). The PCR conditions were optimised at

various temperatures, MgCl₂ concentrations and dNTP concentrations for these primers. A 25µL reaction was initiated using 100ng of template DNA, 1X assay buffer, 0.8 -1mM dNTP, 1-2mM MgCl₂ and 1U *Taq* DNA Polymerase. PCR amplification was performed using thermal cycler with one cycle of 5 min at 94°C, 35 cycles of 40 seconds at 94°C, 40 seconds of annealing temperature, 1 min at 72°C and a final extension of 8 min at 72°C. The PCR amplicons were separated on 2% agarose gel stained with ethidium bromide and were observed on Gel Documentation System (Genesnap, SynGene). The amplicons obtained among all the plantlets were scored in a binary matrix, where 1 (present) denoted the presence of reproducible polymorphic band and 0 (absent) as no amplification. Each character (Band) was treated independently and only consistent, distinct, detectable and reproducible bands in the range from 200 to 1200 bp were considered for scoring and genetic similarity estimation.

2.4.Data Analysis

The data obtained from both the ISSR and RAPD amplicon profiles individually and collectively were subjected to similarity matrix construction using the Jaccard's similarity coefficient (JC) [10]. The neighbour-joining clustering was done on the basis of Jaccard's similarity coefficient calculated based on the binary data using the SPSS (14.0) software.

3. RESULTS AND DISCUSSIONS

Maintenance of genetic fidelity is the most important requirement in micropropagation of any crop species. Existence of somaclonal variation amongst *in vitro* raised plants is a major problem. Molecular markers because of their simplicity have been widely used for the evaluation of genetic stability of micropropagated plant [14].

3.1.RAPD and ISSR Analyses

All the callus and plantlets of different age showed similar RAPD profiles as compared to that of the mother plant. A total of 15 random decamer primers were initially screened, out of which 6 produced clear and scorable amplification products. Each primer produced a unique set of amplification products.

These selected RAPD primers yielded a total of 52 scorable bands (average 8.6 bands per primer) ranging in size from 100 bp (OPA-04) to 1000 bp (OPL20). Number of bands for each primer ranged from 4 (OPA 18) to 10 (OPC 20) (Table 1). In case of ISSR a total of 26 scorable bands (average 5.45 bands per primer) were obtained. Number of bands varied from 6 (ISSR 11) to 14 (ISSR 4) (Figure 1) and size of the bands ranged from 100 bp to 950bp (Table 2).

In the present study although minor variations were detected in terms of band intensity but the number and size of the bands were similar in mother plant, callus and plantlets of all age. Sreedhar *et al.* (2007) [15] has also reported genetic fidelity of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia*) using RAPD and ISSR.

RAPD profiles indicated uniformity among callus of all age and all the plants of different stages of micropropagation, as observed in the form of 80% monomorphic banding patterns (Figure 2). The very low polymorphism observed in the present study could be attributed to the process of dedifferentiation and redifferentiation taking place during callus organogenesis. Similar to our

results calli-derived plants showed uniform banding pattern with the mother plants in *Rehmannia glutinosa* [16], *Desmodium gangticum* [17]. In contrast, use of explants containing organized meristems has been reported to have low risk of genetic variability [18]. Kaushik *et al* (2015) reported clonal uniformity during RAPD analysis of micropropagated *Ophiorrhiza mungos* plantlets [19]. Carvalho *et al.* (2004) used RAPD fingerprints to study genetic stability of *in vitro* propagated plants of chestnut hybrids and no polymorphism was detected among *in vitro* plantlets maintained for 4 years in cultures [20].

3.2.Construction of Dendrogram

The dendrogram tree showed the genetic relationships among the mother plant, callus and plantlets of *B. prionitis* L. based on ISSR and RAPD analysis. On the basis of ISSR analysis the dendrogram classified the samples into two main groups. The first group was classified into two sub-group, where the first one included the mother plant, one month old callus, one month old established culture and rooted plantlet, while the second sub-group comprised of 2 month old callus, 5 month old callus and multiplication phase shoot culture. The second group included hardened plant, 3 and 4 month old callus which were considered as the most distant ones from the other plantlets and their mother plant (Figure 3). In case of the dendrogram based on RAPD analysis the samples were classified into two main groups. The first group was classified into two sub-group, where the first one included five samples viz. 1, 2 and 4 month old callus and rooted plantlet, while the second sub-group comprised mother plant, 5 month old callus and multiplication phase shoot culture. Finally the second group included only the three samples (3 month old callus, hardened plant, one month old established culture) which were considered as the most distance ones from the other plantlets and their mother plant (Figure 4).

Primers	Abbreviation used in the study	Total number of amplified fragments	Fragment size range (bp)
OPA 04	R2	9	110-650
OPA 09	R3	10	300-850
OPA 18	R4	4	300-950
OPC 02	R6	9	350-600
OPC 20	R10	11	200-900
OPL 20	R12	9	180-1000

Table 1: RAPD analysis of genomic DNA extracted from micro-clones of *B.prionitis* using six random decamer primers

Primers	Total Number of amplified fragments	Fragment size range (bp)
ISSR primer 3	7	110-950
ISSR primer 4	14	120-700
ISSR primer 11	6	250-650
ISSR primer 12	7	100-800

Table 2: ISSR analysis of genomic DNA extracted from micro-clones of *B. prionitis* using four ISSR primers

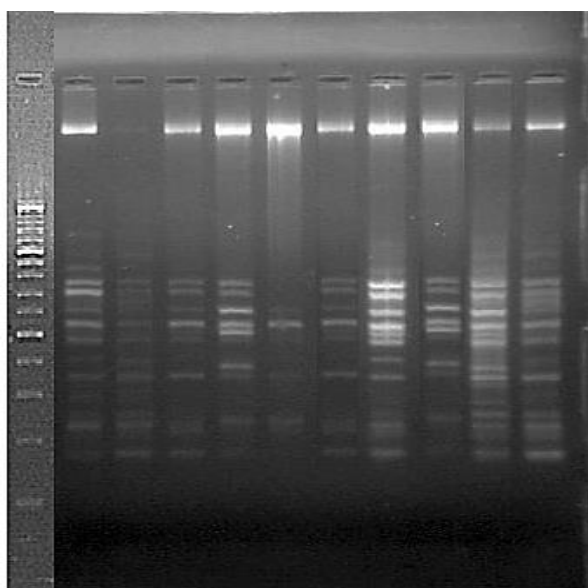


Figure 1: DNA profile obtained using ISSR primer I4

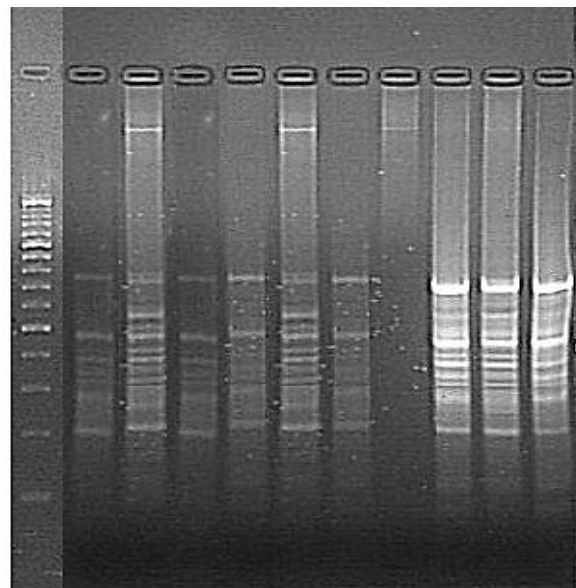


Figure2: DNA profile obtained using RAPD primer R 10

Lane 1 represents a 100 bp molecular marker. Field grown mother plant (Lane 2), Lane 3, 4, 5, 6 and 7 represents Mother plant leaf-derived callus of various age viz. 1, 2, 3, 4 and 5 month old resp. Callus-derived cultures from different stages of micropropagation viz. one month old shoot culture (Lane 8), multiplication phase (Lane 9), rooted shoot (Lane 10) and hardened plantlet (Lane 11).

Dendrogram based on ISSR and RAPD analysis classified the samples into two main groups (Figure 5). The first group was classified into two sub-group, where the first one included 1, 2, 3 and 4 month old callus and rooted plant while the second sub-group comprised of mother plant, 5 month old callus and multiplication phase shoot culture. Finally the second group included only the two samples (hardened plant, one month old established culture) which were considered as the

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most distance ones from the other plantlets and their mother plant. However, the dendrogram reflected the genetic relationships among these propagated plantlets and considered as a good tool to select the plantlets closest with respect to their mother plant. Therefore, these results revealed that ISSR and RAPD represent an efficient tool for estimating the genetic stability and the genetic relationships among the tissue culture derived plantlets.

*****HIERARCHICAL CLUSTER ANALYSIS*****

Dendrogram using Single Linkage

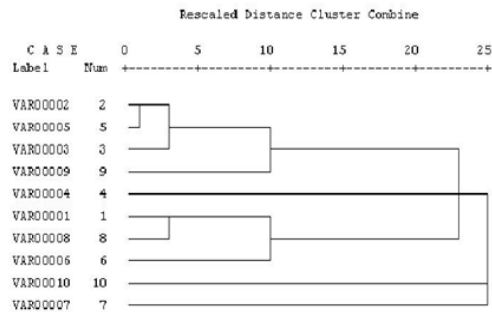


Figure 3: Dendrogram based on ISSR analyses

Dendrogram using Single Linkage

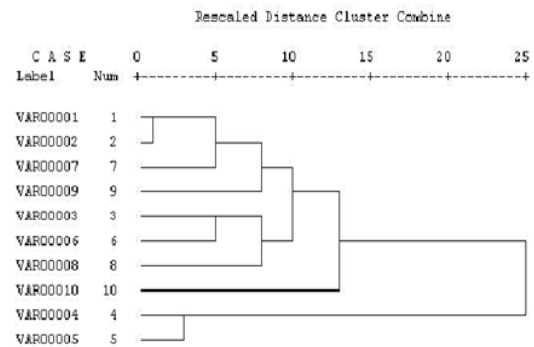


Figure 4: Dendrogram based on RAPD analysis

*****HIERARCHICAL CLUSTER ANALYSIS*****

Dendrogram using Single Linkage

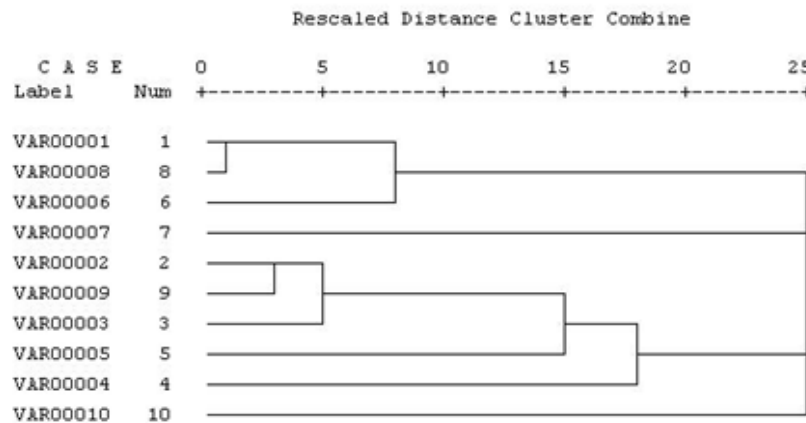


Figure 5: Dendrogram based on RAPD and ISSR analysis

As seen in all the dendrogram the distance between the samples is not more than 25% and so it can be said that there is 75% similarity between the samples with the mother plant. In some cases the distance between the samples is only 2-3%. More number of primers needs to be screened and utilized to cover maximum section of the genome in order to conclude on the genetic fidelity of tissue culture plant. The RAPD markers and ISSR primers target different regions of the genome

and so different dendrograms were obtained with each marker system.

4. CONCLUSION

RAPD and ISSR markers demonstrated nearly monomorphic banding pattern in DNA of mother plant, callus and callus derived plantlets indicating low level of genetic heterogeneity amongst them. Therefore, tissue culture through callus organogenesis can be a good alternate method for the conservation of *B. prionitis* L.

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6. CONFLICT OF INTEREST

The authors hereby declare that they do not have any conflict of interest for the research work communicated through this paper.

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A Study on Microbial Pigment Isolated from Beetroot

D. NAGLE, P. UCHGAONKAR, S. SINGH and D. DASGUPTA*

School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University, Plot No 50,
Sector 15, Belapur, Navi Mumbai, Maharashtra, India.

ABSTRACT: Synthetic colours are harmful to the environment and also have many side-effects. Hence, there is an increasing interest in pigmented microbes as an alternate source of synthetic colorants. The pigments extracted from these microorganisms have diverse applications in the fields of agriculture, textiles, food industries and dyes. The present study deals with the isolation and characterization of a pigmented microbial isolate obtained from Beetroot. The yellow pigment producing microorganism was identified using various biochemical tests. Maximum growth and pigment production was seen when the isolate was allowed to grow in King's B medium at pH 7.0 incubated at room temperature for 96h under shaker conditions. The pigment extraction was carried out by using methanol as a solvent and the absorption maxima of the extracted pigment was determined using UV-VIS spectrophotometer. The pigment exhibited significant antibacterial activity against *S. aureus* and *S. lutea*. It also exhibited significant antioxidant activity as estimated by DPPH assay. The present study can thus be an important lead for use of microbial pigment as an antibacterial as well as an antioxidant agent.

KEYWORDS: Beetroot, Pigments, antibacterial, antioxidant

***Corresponding Author: Dr. Debjani Dasgupta**

School of Biotechnology and Bioinformatics, Dr. D. Y. Patil Deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: drdebjanid@dypatil.edu

1. INTRODUCTION

Colour is an integral part of both – human culture and in human life. It determines not only the acceptance of the product but also provides an attractive appearance to marketable products like food, textiles and pharmaceutical products [1]. The use of pigments as colouring agents has been practiced since prehistoric times. The first synthetic colour was developed by Sir William Perkin in 1856 and since then synthetic colours have been favoured due to their ease of production, cost effectiveness and the fewer amounts required for colouring [2]. However, the effluents released in the processing of these synthetic colours pose a serious threat to the environment. Many of these colours have also been banned due to their allergic reactions, carcinogenicity and other toxicological problems. These adverse effects of synthetic colours have triggered intense research on natural colours and dyes. Natural colours are generally extracted from fruits, vegetables and bacteria which are often referred as “bio-colours” due to their biological origin [3]. The main sources for natural bio-colours are generally plants and microorganisms. The use of plant pigments has many limitations such as non – availability throughout the year, pigment stability and solubility, structural complexity of pigment bearing tissue and the loss of plant species in large scale pigment

production. In contrast, bacteria are a readily available alternate source of pigments. They have enormous advantage over plant pigments including easy and rapid growth in low cost medium, easy processing and growth independent of weather conditions [4].

The beetroot is the tap root portion of the beet plant and is extensively used for its food colouring and medicinal properties. Betanin is the non toxic, water soluble pigment responsible for the deep red – magenta colour of the beet. However, this compound is not broken down in the body and in higher concentrations may cause a condition called beeturia [5]. This crop has a biennial life cycle but only the first year is important for sugar production as the plant produces 18% sucrose. This concentration decreases further due to pathogenic fungi associated with the plant. Hence, most of the studies pertaining to beetroot have been restricted to fungi [6] and to exo-polysaccharide in cases of bacteria [7]. The aim of the current study was to isolate pigmented bacteria associated with beetroot and further explore its application as an antibacterial and anti-oxidant agent.

2. MATERIALS AND METHOD

2.1. Screening and isolation of bacteria from Beetroot

Fresh *B. vulgaris* (beetroot) samples were collected from the local market of Thane, Maharashtra, India. They were bought to the laboratory immediately and cleaned with distilled water to remove traces of soil. The beetroots were then smashed with 10ml of sterile distilled water by using sterile mortar and pestle to extract the pulp. This pulp was then filtered through sterile muslin cloth and the extracted juice was used for screening of pigmented bacteria. This juice was serially diluted using sterile saline and different bacteria were isolated on sterile Nutrient agar plate of pH 7.0 using the spread plate method. Morphologically distinct pigmented colonies were isolated, purified and maintained on sterile Nutrient agar slants until further use [8]. The colony exhibiting maximum pigment production was selected for further study.

2.2. Identification of the pigmented isolate

The isolate exhibiting prominent non- diffusible pigment was identified based on morphological and biochemical tests. The colony characteristics of the isolate on Nutrient agar plate were studied followed by its Gram reaction. The isolate was further identified based on specific biochemical tests using Bergey's manual of Bacteriology. MALDI-TOF analysis was performed to finally confirm the pigmented isolate [9].

2.3. Study of pigment production in different media

Three different media namely Nutrient broth, Milk broth and Kings B broth were used to study the growth of the isolate and enhanced pigment production [10]. 2% of the culture (0.1 O.D at 660 nm) was inoculated in each of the above media and incubated at room temperature (RT) for a period of one week. 10ml aliquot was removed each day for growth curve studies and for determination of amount of pigment produced.

For extraction of pigment, the broth was centrifuged at 6000 rpm for 15 minutes. The cell pellet exhibited colour indicating the intracellular nature of the pigment. Hence the cell free supernatant (CFS) was discarded and the pellet was used for extraction of the pigment. The pigment was

extracted using minimum amount of methanol (99.8%), vortexed until the pellet appeared colourless and maximum pigment was extracted in methanol. The colourless pellet was separated by centrifugation at 10,000 rpm for 10 mins. The extracted pigment filtered and the optical density (O.D) was checked at 472nm using UV-Vis Spectrophotometer [10].

2.4. Effect of physical conditions on pigment production

The media exhibiting maximum growth and pigment production were selected for further study. The effect of physical parameters namely temperature, pH and effect of agitation were studied. The pigment production was studied at temperatures ranging from 4°C to 55°C. The range of the pH selected was from 4 – 10. The effect of agitation was studied by evaluating the pigment production under shaker and static conditions [11]. All these parameters were studied for a period of one week. Aliquots were collected after every 24h and the pigment production was determined as mentioned earlier.

2.5. Antibacterial and antioxidant activity of the extracted pigment

The extracted pigment was checked for its antibacterial activity using the agar well diffusion method. The test organisms used for the study were Gram positive isolates namely *Staphylococcus aureus* and *Sarcina lutea* and Gram negative isolates like *Escherichia coli* and *Klebsiella pneumonia*. 50µl of the extracted pigment was added in each of the well with methanol as the control [12]. Positive control was maintained by adding 50µl Ampicillin (10 mcg/ml) while sterile distilled water served as negative control. All the plates were incubated at 37°C for 24h.

The antioxidant nature of the extracted pigment was determined using the DPPH method. Ascorbic acid (10mcg/ml) served as standard. Methanol was used as control and the % scavenging activity was calculated using the formula:

% scavenging activity = $[(A_0 - A_1) / A_0] \times 100$ where A_0 is the absorbance of control and A_1 is the absorbance of test measured at 517nm [13].

3. RESULTS

3.1.Isolation and identification of the pigmented isolate from beetroot

Isolated colonies were observed on five fold dilution of the beetroot juice. Around 14% of the colonies were pigmented of which morphologically distinct yellow colour colony designated as BY1 was selected for further study. The colony was selected based on its significant growth on nutrient agar plate (Fig 1) as compared to other pigmented bacteria.

The isolate BY1 was identified on the basis of morphological and biochemical characters and further confirmed using MALDI – TOF analysis. The isolate BY1 was identified as Gram positive cocci in clusters, with a non diffusible yellow colour pigment, opaque and elevated colony with regular margin. The colonies exhibited mucoid consistency. The biochemical characterization revealed the isolate BY1 to be positive for catalase and urease. The isolate showed fermentative mode of metabolism for glucose and mannose without gas production. The isolate exhibited negative test for other sugars like lactose, sucrose, mannitol and xylose. The isolate was also

negative for gelatinase, nitratase and citratase activity. The isolate also exhibited a negative methyl red and Voges Proskauv test. All these results indicate that the isolate belongs to *Micrococcus* genus. The MADI-TOF analysis of the culture BY1 is presented in Figure 2.



Figure 1: Pure culture of the isolate BY1 exhibiting yellow colony on Nutrient agar plate

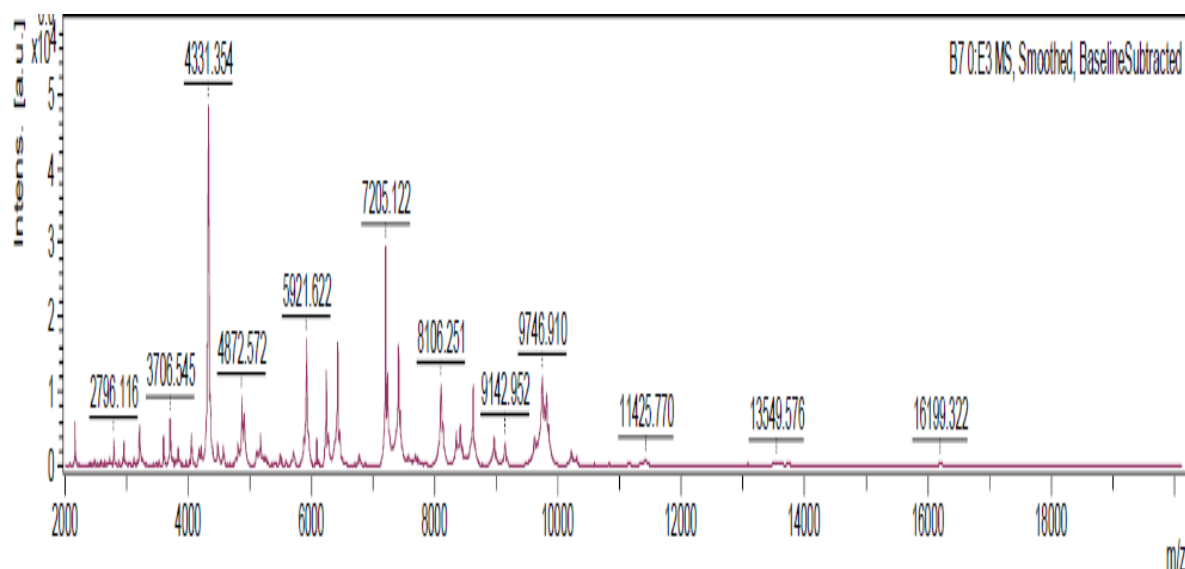


Figure 2: MALDI-TOF profile of the isolate BY1

3.2.Effect of different media on pigment production

The pigment production in three different media namely Nutrient broth (NB), Milk broth (MB) and Kings B broth is depicted in Figure 3. Maximum pigment production was exhibited in Kings B broth and hence this medium was considered for further studies.

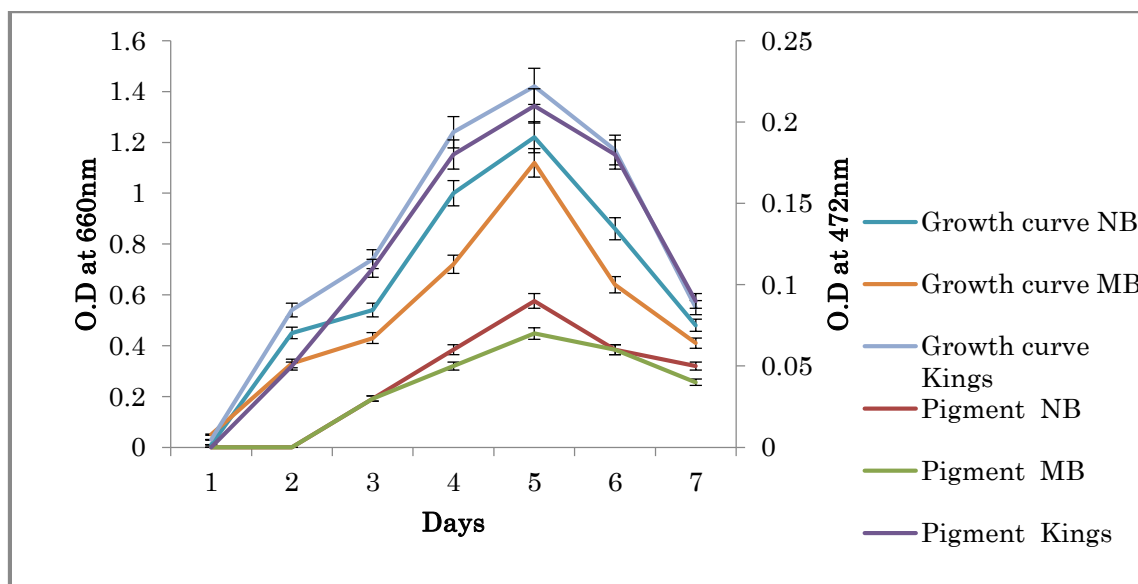


Figure 3: Growth curve studies and pigment production by the isolate BY1 in different media for 7 days of incubation at room temperature

3.3.Effect of physical conditions on pigment production:

The pigment production by the isolate BY1 was studied over a range of pH from 4 to 10 in Kings B medium. However the isolate exhibited growth in the range of pH 6 – 8. Maximum growth as well as pigment production was observed at pH 7.0. The effect of temperature was studied on the pigment production by the isolate BY1. The isolate exhibited growth in the range of 25 - 40°C. Maximum growth and pigment production was observed at 25± 2°C. To study the effect of agitation, growth and pigment production was studied under shaker and static conditions. It was found that shaker conditions exhibited better pigment production as compared to static conditions (Figure 4).

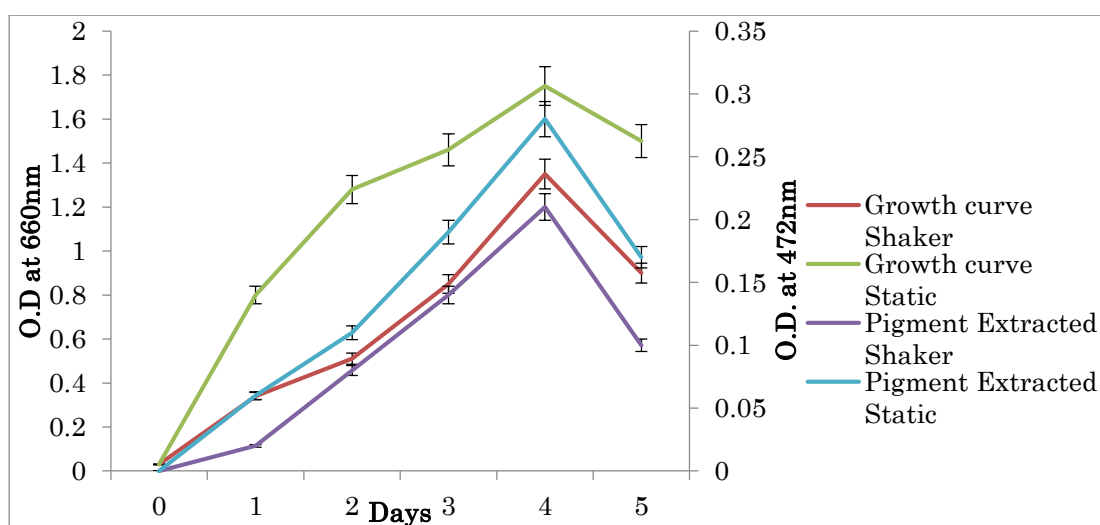
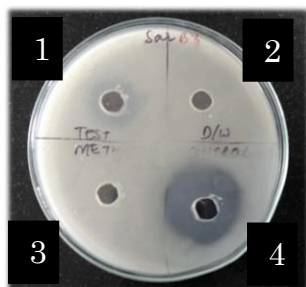


Figure 4: Effect of shaker and static conditions on growth and pigment production for the isolate BY1 in Kings B medium

3.4. Antibacterial and antioxidant activity of the extracted pigment:

The pigment extracted from the isolate BY1 exhibited antibacterial activity only against *S. lutea* (17 mm $\pm$ 1.0) and no inhibition was observed against all the three test organisms selected for study.



Well 1	Extracted pigment
Well 2	Negative control (D/W)
Well 3	Methanol
Well 4	Positive control (10 mcg/ml Ampicillin)

Figure 5: Antibacterial activity of extracted pigment against *S. lutea* after 24h of incubation at 37°C

The antioxidant activity of the extracted pigment was estimated by DPPH assay and it exhibited 90% scavenging activity.

4. DISCUSSION

The use of colours in textile and food industry is practiced since ancient times but isolation of pigments from bacteria is a recent approach. Most of the pigments till date have been extracted from plants and some from animals [4]. Recently, the focus of pigment extraction from plant and animal sources have shifted to pigments extracted from microbes due to their ease of extraction, cheaper production, higher yield using strain improvement and independent of seasonal variation [14]. Hence in the current study, an attempt was made to isolate pigmented bacteria from beetroot samples. The percentage of pigmented bacteria was few in beetroot (14%) which exhibited yellow and orange pigment production. The possible reason could be the presence of a large number of biologically active compounds that lead to decrease in the microflora on beetroot [15]. The morphological, biochemical characteristics and Gram's nature of the isolate suggest that the isolate could be *Micrococcus luteus*. This was further confirmed using MALDI-TOF analysis. Based on the protein profiling studies with Bruker Biotyper database, a score of 2.152 confirms the isolate as *Micrococcus luteus* [16]. The occurrence of *Micrococcus luteus* in Spinach phyllosphere has also been reported by Sharma and Saharan [17].

The use of cold methanol for extraction of microbial intracellular metabolites is considered as an efficient method as it minimizes cell leakage of intracellular metabolites in the extracellular medium during quenching [18]. As depicted in Figure 3, maximum growth and pigment production was exhibited in Kings B broth. Kings B Broth contains glycerol as the carbon source that is known to enhance pigment production. Similar results have been reported for the isolation and identification of chromogenic bacteria from various sources [19]. Maximum pigment production was observed in Kings B broth at neutral pH under shaker conditions incubated at room temperature for 96h. Our results are in accordance with Sorathiya and Shah [20] that report maximum pigment production for *S. marcescens* in the late stages of growth indicating that pigments are secondary metabolites. The pH of the culture medium plays a key role in pigment

synthesis. Our results indicate that biomass and pigment production are significantly ($p < 0.05$) affected by change in pH of the medium. Shaker conditions not only provide oxygen to the culture but also lead to improved homogeneity of other growth factors and metabolic products through mixing leading to enhanced growth and pigment production. The crude extracted pigment exhibited significant antibacterial activity against *Sarcina lutea* but did not inhibit other test organisms selected for study. This is in contrast to the results by Umadevi [21] that report broad spectrum antibacterial activity of marine *Micrococcus* sp. A possible reason could be the stress in marine environment that lead to the production of specific metabolites that exert antibacterial activity. There are a plethora of reports indicating the antioxidant properties of bacterial pigments [22]. In this study, we report the antioxidant properties of the pigment extracted from *Micrococcus luteus*. The free radical scavenging activity of *Micrococcus luteus* along with standard ascorbic acid was determined by DPPH assay. The methanolic extract exhibited a significant DPPH scavenging potential which may be due to the presence of phenolic or flavonoid compounds [23]. The preliminary results of this study suggest that the pigmented extract can be characterised further for use as an antibacterial or antioxidant agent.

5. CONCLUSION

In the current study, pigmented bacteria were isolated from beetroot samples. The isolate exhibited yellow colour pigment which was enhanced on Kings B medium. The methanolic extract of the pigment exhibited specific antibacterial and antioxidant activity. This study can thus prove as a useful lead in the search for a natural colourant. However, further purification and characterization is required to explore the potential of this pigment in medicine, food and textile industry.

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7. CONFLICT OF INTEREST

The authors hereby declare that they do not have any conflict of interest for the research work communicated through this paper.

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Ethyl Cellulose Edible Coatings to Reduce Oil Uptake in Fried Products

M. BHATKAR, R. KALHA, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: Ethyl Cellulose colloidal formulation was used in the coating to reduce oil uptake in deep-fried Indian bread (Dough discs). The use of edible coating colloidal agents can be an effective way to reduce oil absorption in fried products. The effect of 0 mg/ml, 10 mg/ml, 20 mg/ml, 30 mg/ml and 40mg/ml ethyl cellulose solutions considered as a coating formulation for deep fried Indian bread. Ethyl cellulose treated deep-fried Indian bread were fried and analyzed for oil uptake, moisture, and sensory analysis. The oil content and moisture content of deep-fried products were determined using standard AOAC method whereas sensory analysis carried out by a 9-point hedonic scale from 55 untrained panelists. The significant oil reduction observed with increasing concentration of ethyl cellulose concentration. The coating with 30mg/ml and 40 mg/ml ethyl cellulose solution on dough significantly reduced oil absorption in deep-fried Indian bread by 37.50% and 38.59% respectively as compared to control without rejection from the sensory panel. The water retention also increased up to 3.01%.

KEYWORDS: Edible coatings, Ethyl Cellulose, Deep-fat frying, Oil absorption, Water retention

***Corresponding Author: Mr. Ashish Dabade**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Frying is a mass transfer method in which heat and mass transfer take place to carry out desirable changes in the product. Poori (Indian Fried Bread) is Indian traditional food. It's an oil fried 3-4 inch diameter flats of wheat dough [1].

Fried foods have high caloric value with a distinct taste, pleasant flavor, good aroma and texture[2]. The high consumption of oil, especially saturated fat, is leading to increased health-related risks such as coronary heart disease (CHD), hypertension and diabetes, and even to the deaths [3]. The health concern issues have become a health concern due to high fat and energy values [4].

Various factors reported affecting oil uptake in fried foods. Factors such as oil quality and composition, the temperature of the oil during frying and time, the composition of the product, moisture content, porosity, initial interfacial tension pre-frying treatment, and crust size affect the oil content in food. Especially at high moisture content, vapor protects the food from oil absorption by creating an overpressure inside the pores [5].

The gelling property of edible molecules can be used to reduce oil uptake and water migration[6]. Hydrocolloids also tested as additive as oil reducers [7]. The fat content can be reduced up to 83.6% by methylcellulose (MC) films [8]. The cellulose derivative like methylcellulose provides

an efficient reduction in oil absorption due to its barrier properties[9].

An edible film is thin layer formation on food. It is covered on a food product or set as a hindrance between the food product and the surrounding and can be expended. [10, 11].

Many hydrocolloids have the ability to reduce oil absorption. Hydrocolloid films are accepted to be oil-resistant due to their substantial hydrophilicity [5, 12]. Some potentiality and applications of edible coatings as an oil barrier are observed [13].

Ethyl cellulose, prepared from wood pulp or cotton, is intended for use as a food ingredient in Grain Products; Vegetables; Fruits; Milk and Milk Products; Legumes; Nuts and Seeds; Fats and Oils; Sugars and Sweet; and Beverages at a level ranging from 0.0075 to 5.0% of ethyl cellulose which is generally recognized as safe (GRAS) [14].

The objective of the present work to reduce oil absorption from deep fried product and optimize the ethyl cellulose coating formulation for better sensory parameters.

2. MATERIALS AND METHODS

Cellulose derivative suspension: Cellulose derivative Ethyl Cellulose gift sample was provided by Asha Cellulose Limited (India). Ethyl Cellulose formulations with 1%, 2%, 3% and 4% w/v in 99.9% pure ethanol were prepared for coating purpose.

Sample preparation and frying conditions: 35% water was added to the dry solid content and kneaded to make the soft pliable dough. Samples of whole-wheat flour (Pillsbury, India) dough discs with 6cm diameter and 0.5 cm thickness were dipped for 1 s in different suspension concentrations. These dipped samples kept in air for 5 minutes to evaporate suspension alcohol content and then immediately fried. Deep fat fryer was used for sample frying. Coated and uncoated samples were fried with sunflower oil (Gemini, India). Oil replacement was carried out after 3 frying batches. In each batch 3 dough discs were fried. The temperature and time for frying conditions were determined based on sensory parameters and product requirement. The frying temperature and time was maintained at 160 ± 5 °C for 3 minute. The uncoated samples treated as a control according to the method mentioned in (Primo-Martin.et al., 2010)

Lipid content analysis: Soxhlet based lipid extractions were carried out for each dried, grounded sample according to AOAC (AOAC, 2000). Soxhlet extractions were carried out for 6 hours with 99.99% alcohol. Oil uptake relative variation% (OU) in the coated product relative to the uncoated one was calculated as follows:

- Oil Uptake (OU) = (Oil content coated/ Oil content uncoated) x 100
- Percentage of oil absorption reduction = $\{(\text{Oil content coated}/\text{Oil content uncoated}) - 1\} \times 100$

Moisture content analysis: The fresh fried samples were kept in an oven dryer at 105°C for 24 h until constant weight to determine the moisture content as per AOAC (AOAC, 2000). Water retention relative variation (WR) in the coated product relative to the uncoated one was calculated as follows:

- Moisture Percentage = $(W1 - W2) / W1 \times 100$
- WR = $\{(\text{Moisture content coated} / \text{Moisture content uncoated} - 1)\} \times 100$

Where W1= Weight of sample before drying; W2= Weight of sample after drying

Sensory analysis: Organoleptic based 9-point hedonic scale sensory analysis was carried out from 55 non-trained panels for acceptability of the dough discs. Hedonic panel analyzed taste, Appearance, flavor, color, crispiness, overall acceptability. 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely.

3. RESULTS AND DISCUSSION

One-way ANOVA analysis: 1%, 2%, 3% 4% ethyl cellulose treated samples showed significant difference with control sample for oil absorption ($P>0.05$). Control sample oil absorption subset is significantly different with other subsets of ethyl cellulose treated sample oil absorption. 3% and 4% ethyl cellulose treated samples showed similar oil absorption effect as both are in same homogeneous subset (Table.1)

Moisture content of control sample was found in different homogeneous subset than ethyl cellulose treated subset. Moisture content of ethyl cellulose treated product was increased significantly. 3% and 4% ethyl cellulose treated samples were found in same subset with consideration of moisture content. Hence, 3% ethyl cellulose treatment can be considered as optimized and effective treatment (Table.1)

Ethyl Cellulose %	Average Oil %	Average Moisture %	Oil uptake (OU) relative variation %	Water retention relative variation (WR) %
0	21.17 ± 0.116^d	17.66 ± 0.01^a	-	-
1	16.19 ± 0.01^c	18.07 ± 0.01^b	-23.90 ± 0.01	2.28 ± 0.02
2	15.33 ± 0.06^b	18.40 ± 0.05^c	-28.28 ± 0.6	3.05 ± 0.01
3	12.36 ± 0.88^a	18.20 ± 0.10^c	-37.50 ± 0.1	3.01 ± 0.01
4	13.16 ± 0.85^a	18.20 ± 0.10^c	-38.59 ± 0.1	3.01 ± 0.03

Table 1: Characterization of Deep Fried Dough Discs with Different Coating Concentrations of Ethyl Cellulose. (N=3)

^a value $\pm$ S.

Effect of coating formulation on oil absorption and water retention: 3% and 4% ethyl cellulose

formulation treatment showed minimum oil uptake than other formulation treated samples (Fig.1). Dough discs coated with 3% and 4% ethyl cellulose formulation can reduce oil uptake by 37.5% and 38.59% respectively (Fig.2). Water retention of ethyl cellulose treated samples were increased by 3.01% in 3% and 4% ethyl cellulose treated samples (Fig.2.)

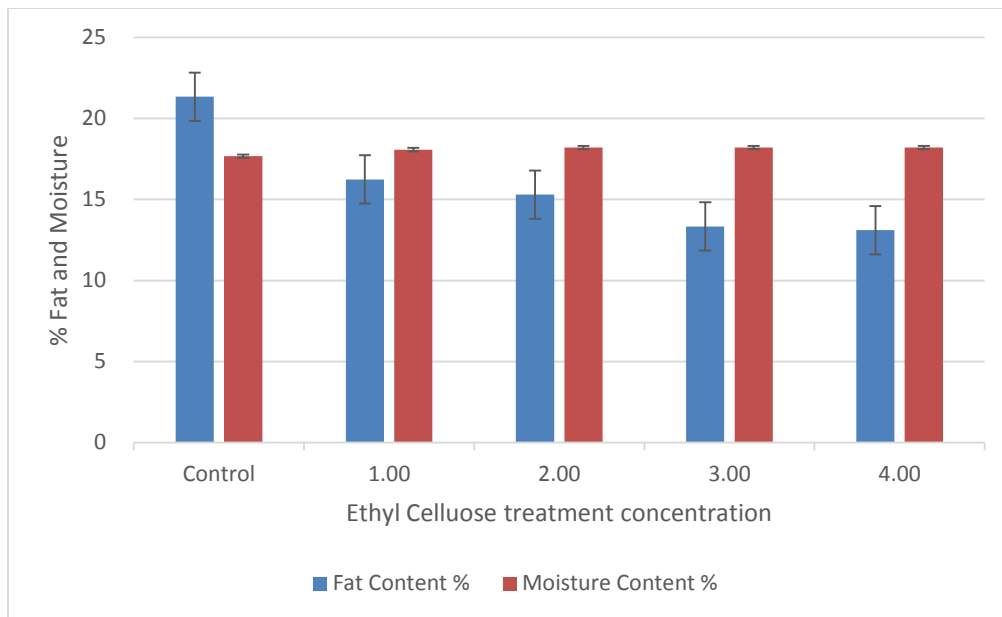


Figure 1: Effect of coating formulation on fat percentage and moisture percentage of coated and control (uncoated) dough discs ($n=3$)

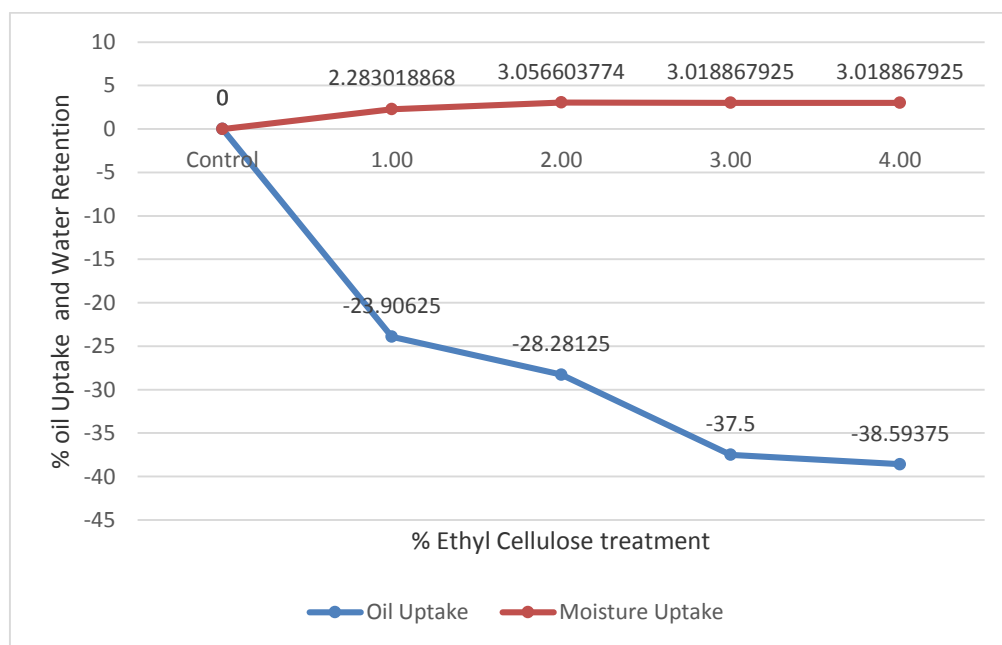


Figure 2: Oil uptake (OU) and Water retention relative variation (WR) in ethyl cellulose coated deep fried dough discs ($n=3$)

Sensory Analysis: Panelist did not reject any sample in sensory evaluation. 3% ethyl cellulose formulation coated flat fried dough sample showed acceptable as a control sample. The graph of 3% ethyl cellulose merged with the control sample (Fig.3.). The overlapping of 3% ethyl cellulose treated sensory evaluation graph with control sample stated that there was no difference in 3% treated sample and control sample. 9-point hedonic scale sensory evaluation based on appearance, color, flavor, taste, crispiness, overall acceptability by untrained panel highly accepted.

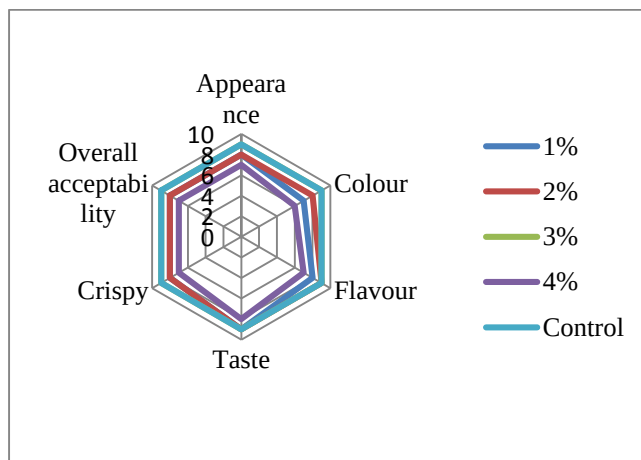


Figure 3: Radar graph for 9-point hedonic scale sensory evaluation based on appearance, color, flavor, taste, crispiness, overall acceptability by the untrained panel. (The graph of 3% ethyl cellulose merged with control sample)

4. CONCLUSION

The coating of 3% and 4% Ethyl Cellulose gum onto dough significantly reduced oil absorption in deep-fried Indian bread compared to control. The 3% and 4% ethyl cellulose can have potential to decrease fat absorption in any deep-fried product.

The 3% ethyl cellulose treated sample were considered as optimized concentration based on oil absorption, water retention and sensory analysis.

At industry level frying is one of the major food processing technique utilized for various fried products. The processing cost can be reduced up to 35% by utilizing ethyl cellulose treatment process.

The 1%, 2%, 3% and 4% ethyl cellulose treated raw material can decrease oil content by 23.90%, 28.28%, 37.5% and 38.59% respectively. Oil absorption reduction up to 37.5% and increase in water retention up to 3.01% can be possible without changing sensory parameters.

Now a day's food product fat content is a major concern for consumers. The application of ethyl cellulose in oil reduction products without changing sensory characteristics can be a successful experiment for snacks segment industry.

5. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Nutraceutical Solution that Alleviates Menstruation Maladies

S. N. MEHTA*, C. HOSKOTE

School of Biotechnology and Bioinformatics, D. Y. Patil deemed to be University, Navi Mumbai, Maharashtra, India.

ABSTRACT: Women during their menstrual periods have to experience various issues such as cramps, fatigue, depression, bloating, stress and mood swings pre-menstrual syndrome, skin inflammation etc. From time immemorial, Ayurveda has an herb which alleviates all the problem mentioned above. This herb is called Shatavari, which has the RDA of 100 grams. However, Shatavari leaves a bitter after taste when consumed. Therefore to circumvent the bitterness of the herb, Chocolate has been used because of its characteristics to mask bitterness and create satiety at a time when women crave for chocolate during such episodes. Chocolate, especially dark chocolate, when incorporated with such ingredients can tend to have a soothing effect on women during their menstrual cycles. The aim of this study is to present the results of a correctly conducted new food product development process which involves development of a center-filled chocolate on the lines of Belgian pralines that can soothe the various issues that women go through during their periods. The research outlines the stages of product development process. It includes: selection and development of the recipe, sensory analysis, shelf life study, consumer response and elements of marketing plan. As the outcome of the food product development process, the prototype of the chocolate was created. The chocolate was made with different ingredients like nuts, flax seeds that have a beneficial role to play in menstruation. The research also highlights benefits of ingredients like Shatavari, hemp seeds, condensed tannins such as pycnogenol and how they can affect the induction of hormones like prostaglandins which are responsible for soothing muscle cramps and controlling inflammation. Presence of vitamins like Vitamin B-6, Vitamin C and Vitamin E can have direct effects on women's skin, quality of their reproductive eggs as well as their moods. A product like this hasn't been introduced in the market since its target crowd can be limited. However, with the right marketing and branding, a product like this could bring a stir in the one dimensional confectionary market which hasn't exactly ventured into healthcare.

KEYWORDS: Menstruation, chocolate, product, development, innovation, food

***Corresponding Author: Ms. Shagun Mehta**

School of Biotechnology and Bioinformatics, D. Y. Patil deemed to be University, Navi Mumbai, Maharashtra, India.

Email Address: m.shagun1997@gmail.com

1. INTRODUCTION

Menstruation, or period, is normal vaginal bleeding that occurs as part of a woman's monthly cycle. Periods usually start between age 11 and 14 and continue until menopause at about age 51. They usually last from three to five days. Besides bleeding from the vagina, women also experience abdominal or pelvic cramping pain, lower back pain, bloating and sore breasts [1].

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During your period, the muscles of your womb contract and relax to help shed built-up lining. Sometimes you'll experience cramps, which are your muscles at work. Food cravings, mood swings and irritability along with headache and fatigue are also some symptoms of menstruation. Premenstrual syndrome, or PMS, is a group of symptoms that start before the period. It can include emotional and physical symptoms. Most, if not all of these problems can easily be cured by Ayurveda and some simple ingredients like nuts and flax seeds. Shatavari is in the wild asparagus family, hence its botanical name *Asparagus racemosus*. Shatavari root is famous for its work in the female reproductive tissue layer. Its cooling, sweet, and unctuous qualities penetrate deeply into the reproductive tissue, promoting fertility, and so much more. This makes it an ideal ally for women's health. Shatavari can be called upon to support a healthy menstrual cycle, aid in a peaceful transition into menopause, and help in the umpteen problems that come with menstruation. The chocolates that we have developed in this study, are essentially a vehicle to carry these ingredients which will prove beneficial to women in many ways during their time of the month.

2. MATERIALS AND METHODS

2.1. Uses and benefits of Shatavari

Shatavari is a species in the family of asparagus which is found in all parts of India, Srilanka, Nepal and the Himalayas. It can also be found in southern parts of China, Africa, Asia and India. It can grow up to 3 feet to 7 feet tall and emerges in rocky soils in piedmont plains which are slightly elevated. This plant is also known as *Asparagus racemosus* [2]. The name 'Shatawari' denotes 'the curer of hundred diseases.' Shatavari has small pine like needles which are called phylloclades which are shiny green and uniform. Shatavari, or *Asparagus racemosus*, has been used for centuries in Ayurveda to support the reproductive system, particularly for females, and as a support for the digestive system, making it an effective supplement for women's health. Shatavari is the herb that is traditionally taken throughout a woman's life, and has been time-tested from the onset of menstruation (puberty) through all phases of menopause. Shatavari is the best herbal tonic recommended for menstrual health. Traditionally, in Ayurveda it is also recommended for increasing the quantity and quality of breast milk. Overall shatavari has various beneficial effects on reproductive organs. It regulates menstrual cycle by balancing hormones like estrogen and progesterone. Generally recommended dose of shatavari is 2-3 grams twice a day with milk. Shatavari can easily cure gastric problems, rheumatism, fevers, headaches and hormonal imbalances. It can also reduce anxiety and stress to a great extent. One of the most important uses of Shatavari is that it helps with mood swings and fertility issues. Consuming Shatavari regularly can also heal respiratory tract problems. It is a natural antibiotic and also acts as a diuretic [3]. Stress and tension can easily be reduced by Shatavari due to its ability to strengthen the immune system. This helps our mind and body to relax and cope up with everyday challenges which might cause tension and anxiety. Mood swings are more common in women than in men. This can occur due to menstruation, pregnancy or hormonal problems. Consuming Shatavari on a regular basis can help to combat mood swings easily. Shatavari is a powerful herb and is full of beneficial ingredients, so by consuming it fertility problems can be kept at bay and the chances of conception becomes high. Antioxidants help prevent free-radical cell damage. They also battle

oxidative stress, which causes disease. Shatavari is high in saponins. Saponins are compounds with antioxidant abilities. According to a 2004 study, a new antioxidant called racemofuran was identified within shatavari root. Two known antioxidants — asparagamine A and racemosol - were also found. Racemofuran, which is found in shatavari, also has significant anti-inflammatory capabilities. Racemofuran acts similarly in the body as prescription anti-inflammatory drugs known as COX-2 inhibitors. These types of drugs are thought to reduce inflammation without serious digestive side effects. Shatavari is used in ayurveda as an immunity booster. According to a 2004 study, animals treated with shatavari root extract had increased antibodies to a strain of whooping cough when compared to untreated animals. The treated animals recovered faster and had improved health overall. This suggested an improved immune response. Shatavari may be one of nature's best kept anti-aging secrets. According to a 2015 study, the saponins in shatavari root helped reduce the free-radical skin damage that leads to wrinkles. Shatavari also helped prevent collagen breakdown. Collagen helps maintain your skin's elasticity. More study is needed before topical shatavari products hit the market [3]. But some researchers believe they may be the future of safe, anti-aging skin care. According to the Anxiety and Depression Association of America, major depressive disorder affects over 16.1 million American adults yearly. Yet many people can't take prescription depression medications due to negative side effects. Shatavari is used in Ayurveda to treat depression. A 2009 study on rodents found the antioxidants in shatavari have strong antidepressant abilities. They also impacted neurotransmitters in the brain. Neurotransmitters communicate information throughout our brain. Some are associated with depression. Regular consumption of Shatavari can help reduce ailments of the respiratory tract and is also helpful in cases of asthma patients. Acts as a diuretic Shatavari helps to fight against urinary tract problems and infections. It also maintains health of the urinary bladder. Apart from this, regular consumption of Shatavari can help to reduce kidney stones in size and sometime cure it completely. Natural antibiotic Often we experience diseases which are a result of certain infection, Shatavari acts as a natural anti-biotic to cure diseases like Staphylococcus, E.coli, dysenteriae and cholera. It also helps to make the immune system much stronger so that it can prevent life threatening diseases from occurring. Uses of Shatavari Shatavari is ideal for people who require stress or anxiety therapy. It increases resistance in both women and men. Shatavari is very useful for relieving menstrual symptoms, improving vision, purifying the blood, improving anti-inflammatory conditions of the body and treating abdominal tumours.

2.2.Uses and benefits of Flax Seeds

Omega-3 essential fatty acids, "good" fats that have been shown to have heart-healthy effects. Each tablespoon of ground flaxseed contains about 1.8 grams of plant omega-3s. Lignans, which have both plant estrogen and antioxidant qualities. Flaxseed contains 75 to 800 times more lignans than other plant foods. Fiber. Flaxseed contains both the soluble and insoluble types. It is one of the richest plant-based sources of omega-3 fatty acids and a whole lot of other essential nutrients [4]. Flax seeds contain just the right amount of fibre, antioxidants and omega-3 fatty acids, and are excellent for your skin, hair and overall health examples are: High in Fiber, but Low in Carbs: flax seeds are high in both soluble and insoluble fibre which can support colon detoxification, fat loss and reduce sugar cravings. Helps in Weight loss: flaxseeds are full of healthy fats and fibre, it will help you

feel satisfied longer so you will eat fewer calories overall which may lead to weight loss. ALA fats may also help reduce inflammation. Improves digestion: The high amounts of fibre aid the digestive process by improving the absorption of nutrients, and also keep the stomach full for longer. Encourages healthy bowel movement: If the inside of your body is performing perfectly, the result will show on your skin. A healthy, proper bowel movement prevents skin diseases and encourages newer healthier skin cells. Flaxseeds Improve Brain Health: Flaxseed is rich in omega-3 fatty acids. Specifically, it's high in a type of omega-3 called alpha-linolenic acid (ALA), which has been strongly linked to brain health. Keeps skin healthy: The omega-3 fatty acids in flax seeds minimize skin rashes, irritation and redness, and also help wounds heal faster. Stops hair breakage: Eating flax seeds makes your hair elastic and strong, and prevents hair breakage.

2.3.Uses and benefits of Dark Chocolate

It will satisfy cravings, help relax muscles, and keep women happier without consuming the fat and dairy in other types of chocolate. Dark chocolate satiates junk food cravings and boosts serotonin levels, which in turn helps alleviate depression and fatigue contains a decent amount of soluble fiber and is loaded with minerals. A 100-gram bar of dark chocolate with 70–85% cocoa contains 11 grams of fiber, 67% of the RDI for iron which is lost during menstruation, and 58% of the RDI for magnesium, 89% of the RDI for copper, and 98% of the RDI for manganese and boron which eases cramps. It also has plenty of potassium, phosphorus, zinc and selenium. It also contains stimulants like caffeine and theobromine, but is unlikely to keep you awake at night as the amount of caffeine is very small compared to coffee. Dark chocolate is loaded with organic compounds that are biologically active and function as antioxidants. These include polyphenols, flavanols and catechins, among others [5]. One study showed that cocoa and dark chocolate had more antioxidant activity, polyphenols and flavones than any other fruits tested, which included blueberries and acai berries.

2.4.Ingredients

Butter 100 g	Cocoa Powder- 3 tbsp
Brown Sugar- 1tbsp	Dark chocolate- 150 g
Golden Syrup – 3 tbsp	Crushed Nuts – 200 g
Flax seeds powder- 25 g	Shatavari powder - 25 g

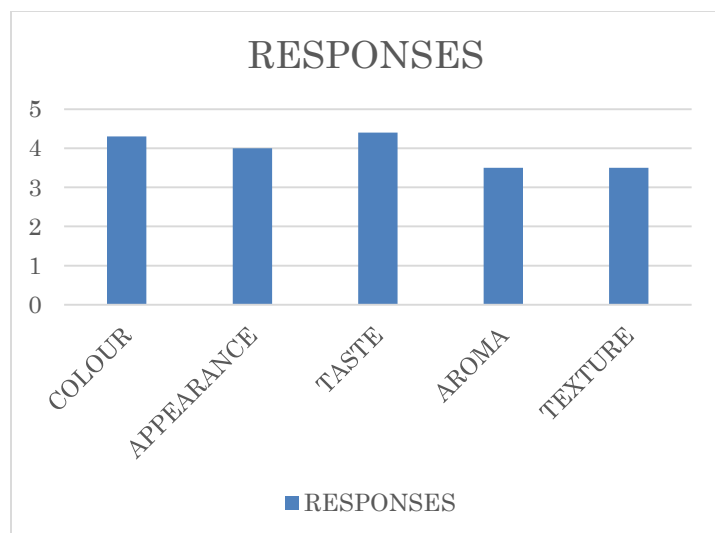
2.5.Method

- 1) Melt butter, sugar, cocoa powder and syrup in a large bowl. Add crushed nuts and mix it with flax seeds powder and shatavari powder and stir well.
- 2) Spread mixture onto a baking sheet so it is about 1.5cm thick. Allow to cool 15 mins.
- 3) Melt chocolate and spread over top. Allow to cool 45 mins and cut into small (3cm) squares

3. RESULTS AND DISCUSSION

3.1.Sensory Analysis

On a 5 point hedonic scale [6] the results were as follows:



5	Extremely Dislike
4	Dislike
3	Neither like nor
2	Like
1	Extremely like

3.2.Shelf Life

The product has a shelf life of 21 days under refrigerated conditions.

4. DISCUSSION

The main reason to conduct this study was to develop a product for women which could solve their maladies concerning menstrual periods. For this product and its intended market, the best promotion strategy would be the pull strategy, where high spending on advertising and promotion is required to build consumer demand [7]. Promoting the product along with health campaigns could be a way of notifying the consumers of its health attributes. Another way of promotion could be by the word of mouth. Women can be given free samples and they could recommend it to other women.

5. CONCLUSION

The results of the realistically conducted food product development process were presented in this study. The research was conducted in a laboratory at a student level and hence did not include any financial indices or industry level processing plans. The presented study allows however, to follow development activities from concept searching and sensory analysis. The process of creating a new product was successful and will be very beneficial to the society in times coming. Women over the years have been using these remedies in one way or another and this product can act as a one stop destination to their menstrual maladies.

6. ACKNOWLEDGEMENT

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We would also like to show our gratitude to Mr. Aakash Vaghani for his insights.

7. CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Extraction and Functional Properties of Crude Proteins from Coffee Silver Skin and its Cost Effective Application

F. JETHWA, P. SHETTY, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, plot no. 50, sector-15, CBD Belapur, Navi Mumbai-400614, India.

ABSTRACT: Coffee silverskin (CS) is the most abundant solid by-product generated during the coffee beans roasting process in coffee industries. This by-product is known to constitute a hazardous environmental problem due to its richness in phytotoxic and/or anti-nutrient compounds. Industries are finding out methods to use it in an economical and effective way. Since this coffee waste contains approximately 18% protein, the present work aimed at extracting crude protein from CS of *Coffea arabica* (*C. arabica*) and *Coffea robusta* (*C. robusta*) and studying its functional properties and application as food additive. Seven different extraction methods were tested in order to optimize the extraction of crude proteins from CS of both the coffee species. Alkaline extraction was found to be the most cost effective method of all wherein isoelectric point of CS crude protein extract was found to be 2.6 for both *C. arabica* and *C. robusta*. This method yielded 1.86 mg/ml and 2.18 mg/ml crude protein in pellets and 0.11 mg/ml and 0.18 mg/ml crude protein in supernatants of *C. arabica* and *C. robusta* respectively even at high temperature. These crude proteins were further used to study functional properties like WHC, OHC, emulsion and foaming activity. WHC and OHC of CS crude proteins of both *C. arabica* and *C. robusta* were found to be greater than soy lecithin. *C. arabica* and *C. robusta* pellets showed 0.67 ± 0.004 and 0.0716 ± 0.004 emulsion activity in 4% emulsion and 0.80 ± 0.004 and 0.83 ± 0.003 emulsion activity in 5% emulsion respectively, which was found to be stable for more than 15 minutes when compared to 0.19 ± 0.003 in 4% and 0.17 ± 0.002 in 5% emulsion of soy lecithin. Maximum foaming capacity as well as stability was observed in supernatants of *C. arabica* and *C. robusta* in 10% concentration (stability upto 30 minutes). Based on these results, it can be stated that crude protein from CS can be easily extracted and used in cheaper way as an additive in food industries.

KEYWORDS: Coffee silver skin, Crude protein, Iso electric point, Emulsion, WHC, OHC, Foaming

*Corresponding Author: Mr. Ashish Dabade

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Coffee which is consumed for its stimulating and refreshing properties is considered to be the third most popular and appreciated beverages after water and tea. As a result of demand for this popular beverage, tons of coffee is being produced all over the globe annually hence resulting in generation of tons of waste from the coffee industry among which, spent coffee grounds (SCG) and coffee

silverskin (CS) are the most significantly generated [1].

CS (present on the green coffee beans as a thin tegument skin) is only by-product produced in large quantity during the coffee roasting process. This by-product is known to constitute a hazardous environmental problem due to its richness in phytotoxic and/or anti-nutrient compounds (e.g. caffeine, polyphenols, and tannins) limiting its direct use in soil or feed applications [2].

Utilizing CS as a source of bioactive compounds for value adding applications is an effective way to minimize their wastage as landfill [3].

It can also contribute to the sustainable development of the coffee chain itself, the global economy and, simultaneously, to a greener environment [4].

Several innovative approaches for utilization of CS has been suggested recently by many researchers, for instance its application in food products such as flakes, bread, biscuits, snacks and as antioxidant beverage for body weight control; in cosmetic products to maintain skin hydration and firmness; and in pharmaceuticals for its anti-glycative and anti-diabetic properties [5].

Utilization of CS as a source of protein has been least explored till date. So the purpose of this work was to extract protein from CS of *Coffea Arabica* (*C. arabica*) and *Coffea robusta* (*C. robusta*) and study its functional properties and evaluate its potential application as food additive.

2. MATERIALS AND METHODS

2.1.Materials

Coffee silverskin samples have been obtained from Subbus Beverages & Foods Pvt. Ltd. in Bangalore, and Soy lecithin purchased from local market was used as control. Hydrochloric Acid (HCl), Trichloroacetic Acid (TCA), Ammonium acetate, Phenol (pH 8), Sucrose, Acetone, Methanol, β -Mercaptoethanol, Phenylmethylsulfonyl fluoride (PMSF), Dithiothreitol (DTT), Glycerol and Folin reagent were purchased from SRL (Mumbai). Sodium Carbonate (Na_2CO_3), Bovine Serum Albumin (BSA), Sodium Hydroxide (NaOH), Copper Sulphate (CuSO_4), Sodium Potassium tartarate, Sodium Dodecyl Sulphate (SDS), Tris base, Ethylenediaminetetraacetic acid (EDTA), Potassium Chloride (KCl), Poly vinylpolypyrrolidone (PVPP) were obtained from HiMedia Laboratories Pvt. Ltd (Mumbai).

2.2.Methods

2.2.1.Preparation of CS powder

CS samples of both *C. arabica* and *C. robusta* (Fig 1a and 1b) were treated with Liquid Nitrogen in a mortar and pestle to minimize proteolysis and other modes of protein degradation. 1 g of coffee silverskin of both *C. arabica* and *C. robusta* when crushed in Liquid N_2 gave 1 g of powder of each (Fig 1c and 1d). The respective CS powders were then transferred in 50ml of Falcon tubes and stored at room temperature separately for further use [6].

2.2.2.Proximate and nutritional analysis of CS powder

100 g of CS powder of both *C. arabica* and *C. robusta* was given to APX Laboratories (Mumbai) for proximate and nutritional analysis. Analysis of energy; carbohydrate; and moisture, ash, protein, fat and crude fibre was carried out [7].



Figure 1a: Coffe silver skin Coffee Arabica



Figure 1b: Coffe silver skin Coffee Robusta

2.2.3.Methods for extraction of CS crude protein extract

Seven different extraction methods namely A, B, C, D, E, F and G, where tested in order to optimize the extraction of crude proteins from CS of both the coffee species.

2.2.4.TCA acetone Precipitation method - Method A

0.2 g of CS powder of *C. arabica* and *C. robusta* was transferred in 2 mL Eppendorf tube, washed with 2 ml 80% acetone and then centrifuged at 10,000 x g for 3 min at 4°C in a Pastcrafts centrifuge. After this treatment the pellet was crushed into fine powder and air dried for 20 minutes. The powder obtained was then rinsed with 10% TCA in acetone for 3-4 times until the supernatant turned brownish to colourless. This preparation was stored at -80°C for further use [8].

2.2.5.Protein extraction by using Phenol Extraction method- Method B

0.2 g of CS powder of both the coffee species was taken in 2 mL Eppendorf tube. A series of washing steps with 2 mL 10% TCA in acetone was performed twice. After each wash the Eppendorf was centrifuged at 16,000 x g for 3 min at 4°C. The tube was again filled with precipitation solution [0.1 M ammonium acetate in 80 % Methanol], vortexed, and centrifuged as mentioned above. The pellet was washed with 80% acetone followed by air drying at 50°C for 10 min to remove residual acetone. Phenol extraction was carried out by mixing pellet with 0.4- 0.8 mL (1:1) phenol and SDS buffer (pH 8). This mixture was incubated for 5 min at room temperature and then centrifuged as mentioned above. Phenol phase was separated and mixed with precipitation solution and kept overnight. The mixture was again vortexed, and centrifuged at 16,000 x g for 5 min at 4°C after which the pellet was washed with 100% methanol and 80% acetone, vortexed, and centrifuged as fore mentioned. The pellet was stored at -20°C for further use [9]

2.2.6. Protein extraction by using EDTA- Method C

1 g of CS powder of both coffee species was taken in a 15 mL Falcon tube. 3 mL extraction buffer [Tris base (6 g) + EDTA (1.8 g) + Sucrose (24 g) + KCl (0.75 g) adjust the pH to 8 and add 2 % mercaptoethanol (0.2 mL in 10 mL D/W) + 1 mM PMSF (0.0017 g in 10 mL isopropanol) and make the volume up to 100 mL] was added and incubated on ice at room temperature for 10 min. Equal volume of Tris-buffer and phenol (pH 8) was added, mixed well and centrifuged at 5,500 x g at 4°C for 10 min. Phenolic phase was collected from the separated phases and then 3ml of extraction buffer was added and similarly centrifuged as above. The top layer of phenolic phase was collected in separate tube; 12 mL of precipitation solution was added to it and incubated at -20°C overnight. The mixture was vortexed, and centrifuged as mentioned above. Pellet was collected, and was washed thrice with precipitation solution and once with cold acetone, vortexed, and centrifuged at 5,500 x g for 5 min 4°C. Final pellet was collected and stored at -20°C for further use [10]

2.2.7. Modified TCA-Acetone precipitation with DTT (Method D)

0.2 g of CS powder was taken in 2 mL of Eppendorf tube. 1 mL of chilled 10% TCA in acetone with 20 mM DTT was added and incubated at 4°C for 15 min. Tubes were vortexed, and centrifuged at 16,000 x g for 15 min at 4°C. Pellet was then washed with 1 mL of precipitation solution, incubated for 15 min at 4°C, vortexed, and centrifuged as mentioned above. Repeat washing with acetone and centrifuge at 16,000 x g for 15 min at 4°C. The pellet was allowed to air dried at room temperature to remove residual acetone. Pellet was resuspended in 1:1 Phenol-SDS buffer (pH 8), incubated at room temperature for 1 hour, then tubes were again centrifuged as mentioned above. Upper phenol phase was collected in another Eppendorf tube and filled the tube with precipitation solution, incubated overnight at -20°C, again centrifuged as mentioned above. Light brownish to white pellet was obtained which was washed with 2 mL 100% methanol and 80% acetone (3 times each) and centrifuged at 16,000 x g for 15 min at 4°C. Final pellet obtained was stored at -80°C with 80% acetone for further use [11].

2.2.8. Modified TCA-acetone precipitation with hot SDS buffer (Method E)

2gm of CS powder was suspended in hot 10-12 mL of 2% SDS extraction buffer [12] and heated at 95°C for 8 min, vortexed, and centrifuged at 8,000 x g for 15 min at 4°C. Protein precipitation was carried out with 30 ml of acetone containing 10% TCA and 20 mM DTT at -20°C for 45 min; centrifuged at 18,000g for 10 min at -7°C. Pellet was then washed with ice-cold acetone containing 20 mM DTT, incubated for 60 min at -20°C, and then centrifuged at 20,000g for 10 min at 4°C and the supernatant was discarded. The protein pellet was air-dried for 5 min then dissolved in rehydration buffer [13].

2.2.9. Alkaline extraction with modified temperatures- (Method G)

0.5 g of CS powder was suspended in 20 ml of 0.1 M NaOH and kept at 250C, 450C, 600C, 800C, 950C, 1100C, 1200C for 2 hours, then centrifuged at 15,000g for 10 min at 40C and supernatant was collected. pH was adjusted using 0.5 M HCl, until it reaches at its isoelectric point (2.6 pH for coffee silver skin) then again centrifuged at 15,000 x g for 10 min at 40 C. Supernatant and pellet

both were collected and stored at 40C until further use [14].

2.2.10. Determination of protein concentration of palet and supernatent

Protein content of all the supernatants and pellets were determined[15][16].

Functional properties of CS crude protein extract

Water holding capacity (WHC) and oil holding capacity (OHC): Water holding capacity (WHC) and oil holding capacity (OHC) of crude protein extracts were determined by mixing 0.5 g of pellet sample with 5 mL of distilled water and sunflower oil (Density=0.92 g/mL) respectively. The mixtures were vortexed for 1 min, centrifuged at 2,330 x g for 30 min at 25°C, and the volume of supernatant was determined. WHC was expressed as grams of water held per grams of sample, while OHC was expressed as grams of oil held per grams of sample [17].

Emulsification activity (EA) and emulsion stability (ES)

Emulsification activity and emulsion stability were determined with slight modifications. Emulsions of various concentrations 1%, 2%, 3%, 4% and 5% were prepared by mixing 0.5 g of CS pellet sample in 5 mL distilled water and then vortexed for 1 min. 5 ml sunflower oil was added to this mixture, and homogenized in a homogenizer (Remi motors Ltd.) at 8,000 rpm for 3 min at 25°C [17,18]. Then 50 µl of emulsion was diluted with 5 mL of 0.1% (w/v) sodium dodecyl sulfate (SDS) solution. The absorbance of the emulsion was measured immediately after emulsion formation using UV-Visible spectroscopy at 500 nm and expressed as emulsion activity of protein [19].

To determine the emulsion stability index, the emulsion was left at 25°C for 10 min, and then 50 µl of emulsion was diluted with 5 mL of 0.1% (w/v) SDS solution and the absorbance at 500 nm was measured [25]. Emulsion stability index was calculated according to the following equation:

$$\text{Emulsion stability (min)} = \frac{A_0 \times t}{\Delta A}$$

Where, A_0 is the absorbance at 0 min

ΔA is the change in absorbance during the time interval (t)

Foaming capacity (FC) and foaming stability (FS)

Foaming capacity and foaming stability were examined for supernatant sample of various concentrations 2%, 4%, 6%, 8%, 10%, 12%, 14%, by dissolved in distilled water. This mixture was whipped at high speed with hand blender and poured into 100 mL volumetric cylinder [22].

$$\text{FC (\%)} = \frac{\text{Volume after agitation-volume prior to agitation}}{\text{Volume prior to agitation}} \times 100$$

The samples were allowed to stand for 30 min at room temperature to estimate the FS given by the formula below,

$$\text{FS (\%)} = \frac{\text{Residual from volume}}{\text{Total foam volume}} \times 100$$

3. RESULTS AND DISCUSSIONS

3.1. Proximate and nutritional analysis of CS powder

CS powder of *C. arabica* and *C. robusta* was found to contain 13.6 % and 18.4 % protein respectively as shown in Table 1.

3.2. Determination of protein concentration by different extraction methods

Fig.2. illustrates concentration of proteins in pellets obtained by different extraction methods. Pellets of both coffee species obtained by method A, C and E showed presence of higher concentration of protein as compared to method B and D. This can be due to presence of purified proteins even in small quantity of pellet. On exposure to air, the pellets turned into brown to blackish colour probably due oxidation of proteins. As these methods yielded very low amount of proteins and required white pellet for further use. These methods were also not economical from industry point of view [9].

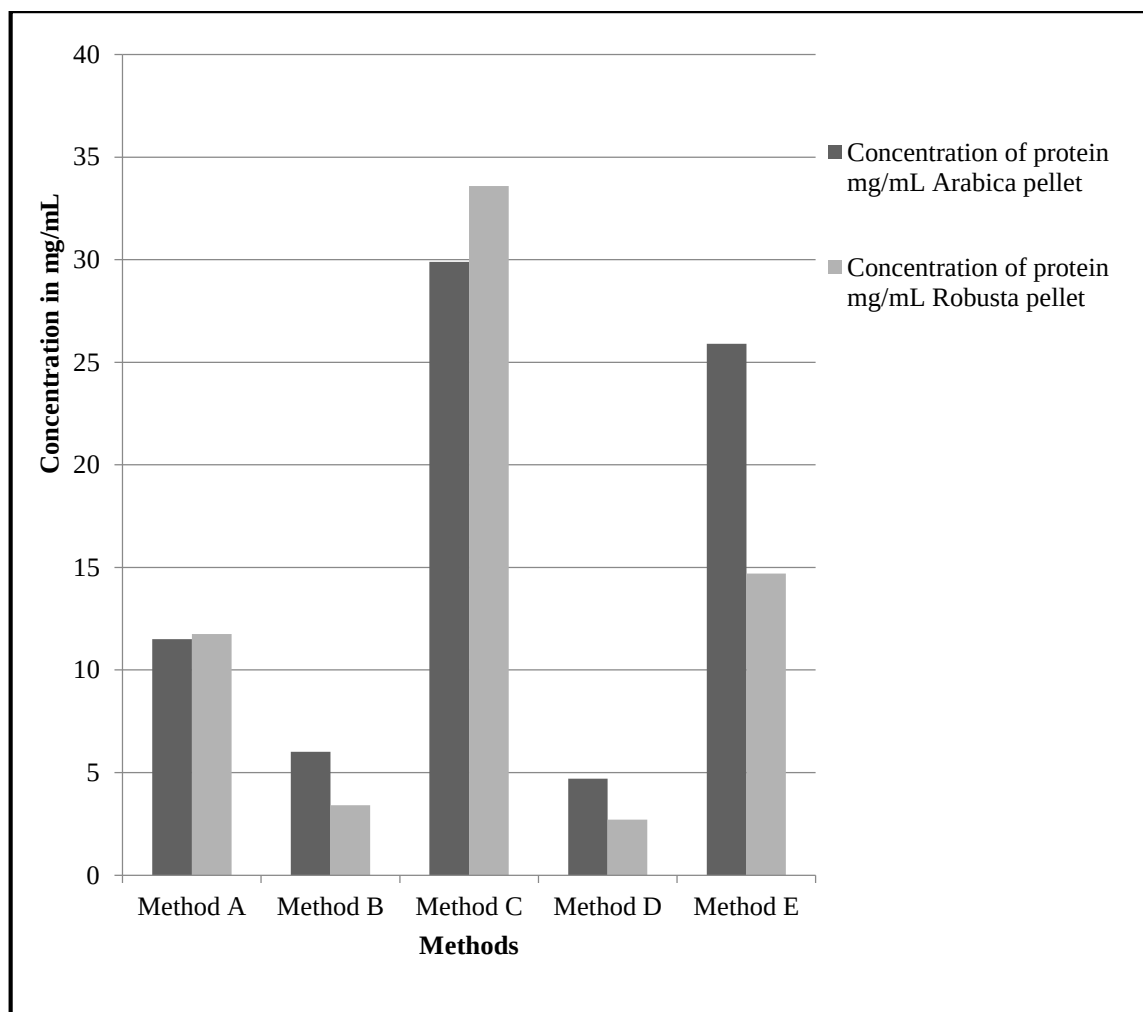


Figure 1: Concentration of proteins in pellets obtained by method A, B, C, D and E

Proteins present in pellets and supernatants of both *C. arabica* and *C. robusta* could withstand high temperature (fig 2a. and 2b.) without getting denatured. As temperature increases concentration of protein also increases in both *C. arabica* and *C. robusta* protein pellets and supernatants. Based on method F and G i.e. alkaline precipitation methods isoelectric point (pI) of proteins present in pellets and supernatants was found to be 2.6. It was found that proteins present in pellets were soluble in nature whereas those in supernatant were found to be insoluble with other components. These methods were further used to carry out to study of functional properties of CS proteins as they are very simple and cost effective and can be applicable in industry point of view [23].

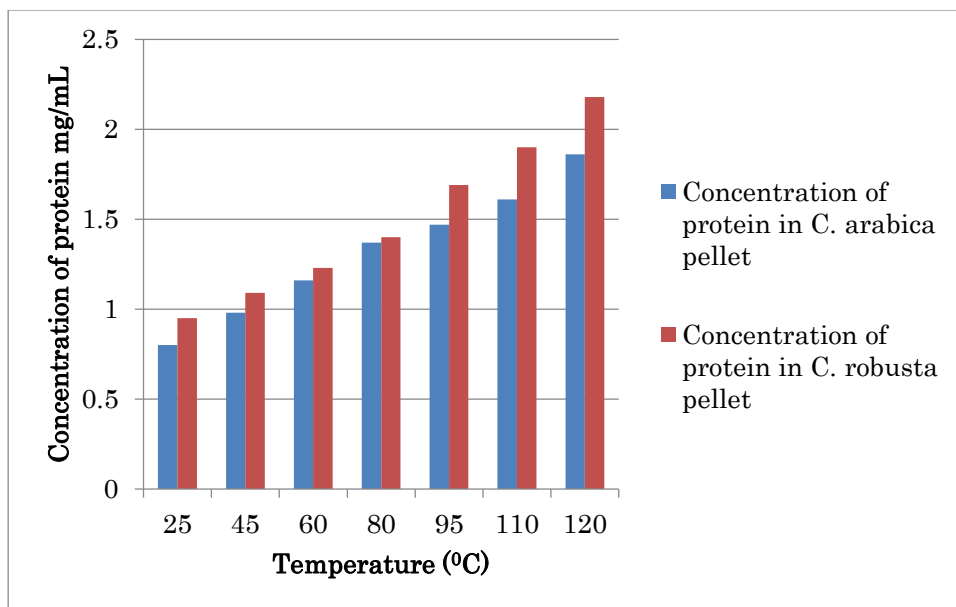


Figure 2a: Concentration of protein in pellets obtained by alkaline precipitation methods.

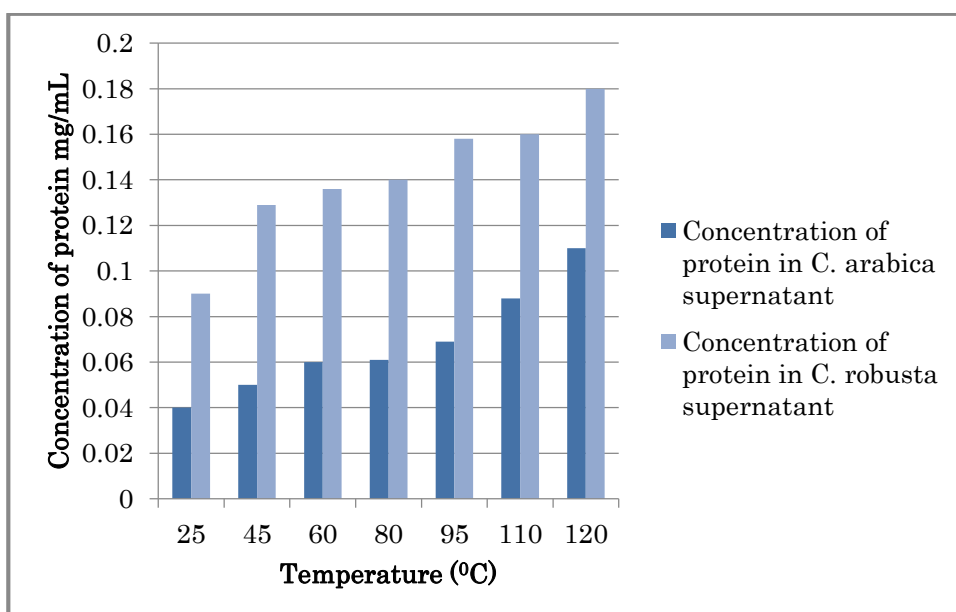


Figure 2b: Concentration of protein in supernatants obtained by alkaline precipitation methods.

3.3. Water holding capacity (WHC) and oil holding capacity (OHC)

The pellets were found to have more oil holding capacity than water holding capacity. It was also found that CS has good WHC and OHC as compared to soy lecithin (Table. 2) indicating its application in stabilization of high-fat products and emulsions. Both WHC and OHC play an important role during preparation, processing, and storage of foods. Moreover, they can influence in the nutritional and sensory characteristics of food and its physical behaviour [30].

3.4. Emulsification activity (EA) and emulsion stability (ES)

For EA and ES protein pellet with more concentration of protein was selected as it showed positive test for emulsification at temperatures ranging from 25°C to 120°C. Table 3 and 4 shows that as concentration of emulsion is increased the EA and ES of pellets of *C. arabica* and *C. robusta* is more as compared to soy lecithin. *C. robusta* pellet was found to be more active and stable than *C. arabica*. This is because the protein fractions that are present aid in binding the fibre to the oil or water interface. This shows proteins extracted from *C. robusta* it can be used in other food industries as an emulsifier that require long emulsion stability. No significant difference was seen in 1% *C. arabica* and *C. robusta* pellets in both emulsification activity and emulsion stability when compared with soy lecithin means both are having same property. Significant difference was seen from 2% to 5% in both in *C. arabica* and *C. robusta* pellets both emulsification activity and emulsion stability when compared with soy lecithin indicating protein extracted CS of *C. arabica* and *C. robusta* have greater EA and ES properties at higher emulsion concentrations [17,25].

3.5. Foaming capacity (FC) and foaming stability (FS)

Supernatant with more concentration of proteins was selected for studying FA and FS at room temperature as it showed positive test for foaming property at temperatures ranging from 25°C to 120°C. Figure 3a and 3b shows that as concentration increases the FA and FS of both *C. arabica* and *C. robusta* supernatants also increases. Proteins act as interface in between air and water and form a film to reduce the interfacial tension which leads to more stability. Hence, there are some proteins in CS which have great foaming activity and stability that allows the foam to be stable for long time [26, 27]. So CS can also be used as foaming material in ice-cream industries, coffee whiteners and other industries by extracting the proteins.

4. CONCLUSION

CS is a waste material from roasting process of Coffee beans and it is very much hazardous to the environment as it rich in anti- nutrient compounds. Thus, industries are finding out ways to use it in an effective way. In this study protein was extracted by various methods and extraction method was found to be the best and cheapest as this method yielded more and good amount of proteins at high temperature, and can also be employed efficiently at industrial level. This protein was then used to study the WHC, OHC, emulsification and foaming. Proteins from CS of both *C. arabica* and *C. robusta* showed good results at higher concentrations. This states that CS proteins can be used in other food as functional additive for instance as foaming agent in ice-cream production or emulsifiers in fat spreads.

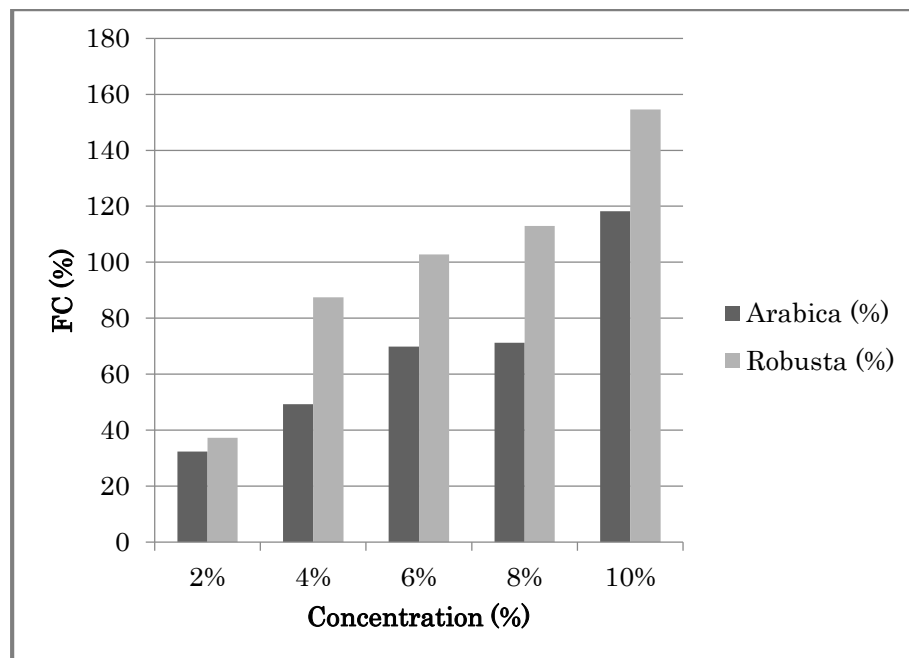


Figure 3a: Foaming capacity of *C. arabica* and *C. robusta* supernatant samples

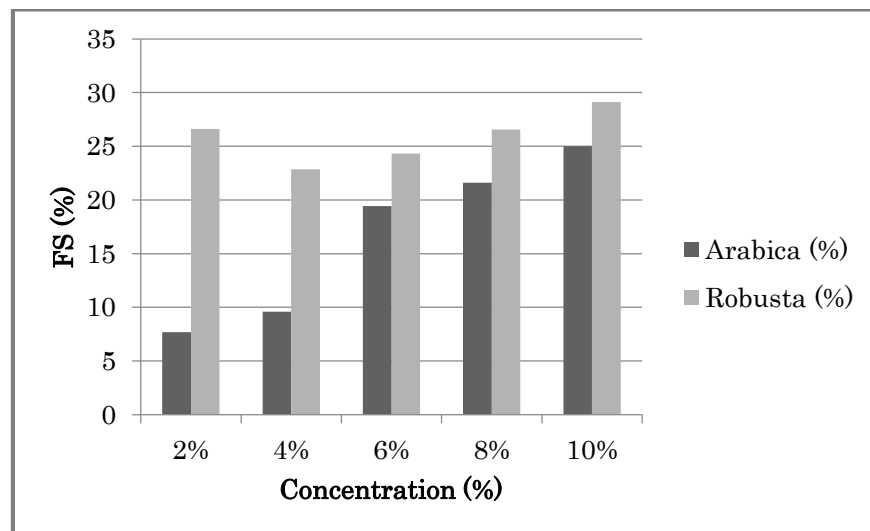


Figure 3b: Foaming stability of *C. arabica* and *C. robusta* supernatant samples

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6. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Extraction of Chlorogenic Acid from Green Coffee Beans for Preservation against Bread Spoilage

A. RAI, R. SHUKLA, S. SAWANT, R. SHETYE, D. BOPTE, H. GAVANDI*

Department of Biotechnology, Annasaheb Vartak College of Arts, Science and Commerce, Vishal Nagar-401202, Vasai West, Maharashtra, India.

ABSTRACT: Coffee is a popular beverage which is extensively used throughout the globe. It is rich in phenolic compound known as Chlorogenic Acid (CGA). CGA is mainly the content of green coffee as it is lost after roasting. A number of studies have concluded the antibacterial activity of green coffee and its by-products. The aim of the study is to extract CGA from green coffee beans to determine its inhibitory potential against bread spoiling pathogens. It was extracted from green coffee by solvent extraction using isopropanol and was analyzed by Folin-Ciocalteu Method using Spectrophotometer. The extracted CGA was tested to determine its Minimum Inhibitory Concentration against bread spoiling pathogens. Test organisms used were – *Escherichia coli* and *Bacillus subtilis* as they are common pathogens and contaminants. From the view of antibacterial assay they were chosen due to their difference in Gram's nature. Antibacterial activity of CGA was tested by using agar well diffusion and paper disc techniques. Its preservative and antifungal nature was tested against white bread slice. The results confirmed that CGA isolated from green coffee beans showed antimicrobial effect against test organisms. It also increases the shelf life of sliced bread by inhibiting mold formation. Thus it is concluded that CGA may be used as a natural and preservative for baked products and also has beneficial properties such as antimicrobial activity. Antioxidant properties, etc. of CGA also helps in weight loss. For comparative studies between brown and green coffee, caffeine, a major content of brown coffee was extracted using dichloromethane. However it was observed to have far less or no antimicrobial activity as compared to CGA.

KEYWORDS: Green coffee bean, Chlorogenic acid, antimicrobial activity, anti-oxidant, caffeine.

Corresponding Author: Mrs. Heena Kausar S. Gavandi*

Department of Biotechnology, Annasaheb Vartak College of Arts, Science and Commerce, Vishal Nagar-401202, Vasai West, Maharashtra, India.

Email Address: heenakausarp@yahoo.co.in

1. INTRODUCTION

Coffee is extremely consumed worldwide. It is noteworthy that today it is considered as a functional food primarily due to its high content of compound that exerts anti-oxidant actions. These salutary effects are mainly due to a magical compound known as Chlorogenic Acid (CGA) which is considered to be a phytochemical, CGA is seen in a number of higher plants like sunflowers, pine-apple, strawberries, blue-berries, etc. However, it is present in higher amount in green coffee beans, especially raw Arabica coffee due to its phenol content. In the previous few decades' usage of phenolic compounds from green coffee as an antimicrobial has been increased.

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Chlorogenic acids comprise a major class of phenolic compounds. It can be used a natural food preservative due to their antimicrobial and antioxidant effects. It mainly contains three components that are – Caffeoylquinic acid (CQAs), Fernloylquinic acid (FQAs) and DiCaffeoylquinic acid (diCgAs). It is an ester of caffeic acid and quinic acid. Basically coffee is of two types – green and brown. CGA is present in higher quantities in green coffee and to a lesser degree in roasted coffee, as it is broken down by roasting process. The degree of destruction increases with temperature and time of roasting with green coffee beans. Roasting destroys 70%-94% of CGAs depending on the way it was roasted.

Addition of synthetic chemical food preservatives is a means to inhibit microbial growth and control food spoilage. However consumers currently worry about such chemical preservatives and the impact it may have on their personal health. As a result, there is an increasing interest in the development of naturally derived antimicrobials as an alternative to synthetic chemical food preservatives. Extracted CGA from the coffee has the potential to inhibit the growth of food spoilage micro-organisms. Also it can be used as a food additive to prolong the shelf life of food. Mold spoilage in bread is a serious issue in bakeries. It is a primary factor in reducing the shelf life of baked products. However, it also depends upon temperature, season and processing method. Chlorogenic Acid can provide a solution to this.

2. MATERIALS AND METHODS

2.1.Extraction of Caffeine using Dichloromethane

Powdered brown coffee was solubilized using Sodium carbonate by boiling it in distilled water. Dichloromethane methane was used to extract caffeine from filtrate. Later, anhydrous Calcium Chloride was added as a dehydrating agent. The extract was heated to obtain tan colored caffeine [1].

2.2.Extraction of Chlorogenic Acid from Green Coffee Beans by Solvent Extraction method using Isopropanol

Crushed green coffee bean was added to a mixture of isopropanol and water formed in the ratio of 70:30. It was kept on rotatory shaker for 5 hr to which 10 ml of this solvent was added after each 30 mins. Ammonium sulphate, petroleum ether and chloroform were added to eliminate other component than chlorogenic acid. Crude CGA was obtained upon air drying [2].

2.3.Estimation of crude Chlorogenic Acid by Folin-Ciocalteu assay using Visible Spectrophotometer (Quantitative analysis)

Crude CGA was estimated by standard protocol of Folin-Ciocalteu assay with some modifications. Its absorbance was then found at 750 nm via UV-Visible Spectrophotometer [3, 4].

Crude Caffeine, Crude Chlorogenic Acid Extract, Crude Chlorogenic Acid Extract diluted with sterile distilled water (1:1), Crude Green Coffee Extract was used as test solutions for CGA sensitivity test. Turbidity of test cultures was adjusted to 0.5 O.D. as per McFarland's Standards.

2.4.Determination of Minimum Inhibitory Concentration

This was done by two fold broth dilution method for both test organisms viz. *E.coli* and *B.subtilis* using sterile Luria Bertini Broth against the extracted crude CGA. These tubes were incubated for 24 hrs at 37°C and negative control at 4°C for observation [5].

2.5.CGA Sensitivity Test by agar – well diffusion

Sterile Mueller-Hinton agar was used for the assay using the mentioned test organisms. Standard protocol was followed for the same [2].

2.6.CGA Sensitivity Test by Paper-Disc / Kirby-Bauer Method

Paper discs of caffeine, green coffee, and CGA extract were made. Sterile Mueller-Hinton agar was used and standard protocol was used [5].

2.7.Antifungal / Preservative Action on Bread Spoilage

Two breads, one with CGA and other without CGA was made moist with the help of distilled water and kept at room temperature. Regular monitoring of mold formation on both the breads was done for 5-6 days [2].

3. RESULTS AND DISCUSSION

0.13 gm of tan colored crude caffeine was obtained from 10 gm of brown coffee powder.

4.7 gm of crude CGA was obtained from 10 gm of green coffee powder. It was further dissolved in 50 ml of distilled water for experimental use.

Concentration of CGA was found to be approx. 1500 µg/ml as its absorbance was 1.7 measured at 750 nm. This was done by extrapolating it on standard calibration curve of CGA [3].

The results of MIC indicated that Chlorogenic Acid inhibited the test organisms which are the causative agents of bread spoilage. The MIC i.e. least concentration which inhibited the growth of test organisms was found to be 32 µg/ml.

In agar well diffusion, the sensitivity of test organism to the crude CGA extract was determined. Both the test organisms were inhibited by CGA. Also its degree of inhibition was similar to both. Along with CGA, caffeine and coffee extract were also tested for their sensitivity. The test solution also included a diluted sample of CGA (1:1). Highest zone of inhibition was observed for crude CGA extract. For *Escherichia coli* and *Bacillus subtilis*, it was found to be same i.e. 30 mm. Lowest zone of inhibition was found for caffeine due to its low inhibitory effect. Even the coffee extract showed good zone of inhibition but less than CGA indicating that green coffee alone has good inhibitory effect. Observation of zone of inhibition in diluted CGA shows that even the diluted form can give good inhibitory effect.

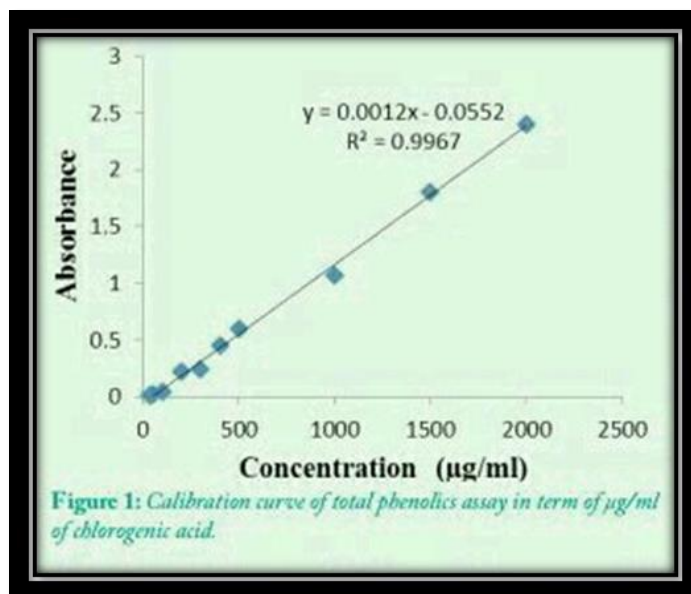


Figure 1: Concentration of crude CGA extract

Conc. (µg/ml)	1024	512	256	128	64	32	16	08	04	02	PC	NC	MC
Growth	-	-	-	-	-	-	+	+	+	+	+	-	-

Table 1: MIC result for *E.coli* and *B.subtilis*

Key: (-) - Growth Inhibited, (+) - Growth Observed

PC,NC,MC – Positive, Negative and Media Control

TEST SOLUTION (crude)	ZONE OF INHIBITION(mm)	
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
Coffee Extract	23	28
Caffeine Extract	12	-
CGA Extract	30	30
1:1 CGA extract	22	21

Table 2: Agar well diffusion

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Among the test samples, CGA showed largest zone of inhibition i.e. 13 mm for *Bacillus subtilis* and 14 mm for *Escherichia coli* for paper disc method. However Tetracycline disc used as control was found to have maximum zone of inhibition due to its ability to inhibit large number of pathogens. In both the test organism, caffeine was found to have no inhibitory effect. For Coffee extract *Escherichia coli* showed some sensitivity while *Bacillus subtilis* was found to be resistant. Thus, we can say that CGA can inhibit growth of food pathogens similar to that of an antibiotic tetracycline.

Bread without CGA showed rope formation after 5-6 days. Acidic nature of CGA prevented any mold growth on bread with CGA.

TEST SOLUTION (crude)	ZONE OF INHIBITION (mm)	
	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
Coffee Extract	-	08
Caffeine Extract	-	-
CGA Extract	13	14
Std. Tetracycline (10 mcg)	24	27

Tables 3: Paper Disc Method



Figure 2: Bread with CGA



Figure 3: Bread without CGA

In the study here, bread slices with and without CGA were monitored daily. On the very next day of CGA application, both the bread slices were as it is. After 2nd day, mold formation was observed to begin on bread slice without CGA. After 5-6 days of incubation, dense mold formation occurred on bread slice without CGA. On the other hand, bread with CGA remained as it is. Thus, CGA was observed to inhibit / delay the mold formation. This shows that the CGA can be used for preserving bread or bakery products against mold contamination.

4. CONCLUSION

Our study shows one of the easiest and cheap methods of extracting Chlorogenic acid from green coffee bean by isopropanol which can be done at lab level. It says that CGA has the capacity to stop the growth of both Gram positive (*Bacillus subtilis*) and Gram negative (*Escherichia coli*) bacteria even in low concentrations. It also exhibits antifungal activity by restricting formation of mold on bread.

Synthetic preservatives like sodium benzoate have the ability to deprive human cells of oxygen, break down immune system and cause cancer. Thus, in the ever increasing quest of searching safe preservative, CGA holds a promise to be the best alternative for synthetic additives that too without any side-effects.

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Comparative Analysis of Various Parameters of Oyster Mushroom Cultivated Using Different Substrate Combination

P. WADKE*, M. PATWARDHAN

KET's V. G. Vaze College, Mulund East, Mumbai, Maharashtra, India.

ABSTRACT: Popularity of oyster mushroom is increasing for its ease of cultivation, high yield potential as well as its high nutritional value. Generally, paddy is used as substrate for cultivation of mushrooms. But we used paper and sugarcane bagasses in combination with paddy and only paddy was kept as control. We chose these substrates as they are waste organic materials which are easily available. After cultivation of mushrooms, we particularly focused on the comparison of the carbohydrates, proteins, antioxidants (phenolic compounds and flavonoids) in mushrooms plucked from each bag. Extract of mushroom was investigated using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and water. The result indicates that paper along with paddy can be a better substrate for enhancing proteins, phenolic compounds, flavonoids and antioxidant activity and also to lower the carbohydrate content.

KEYWORDS: Oyster mushroom, nutritional value, waste organic, paddy

***Corresponding Author: Prajakta Wadke**

KET's V. G. Vaze College, Mulund East, Mumbai, Maharashtra, India.

Email Address: prajakta9697@gmail.com

1. INTRODUCTION

Mushrooms are used extensively in cooking, in many cuisines (notably Chinese, Korean, European, and Japanese). Though neither meat nor vegetable, mushrooms are known as the "meat" of the vegetable world. It is the fleshy, spore-bearing fruiting body of a fungus, typically produced above ground on soil or on its food source. There are edible as well as toxic types of mushrooms. Most widely used mushrooms for edible purposes are white button mushroom (*Agaricus bisporus*) and oyster mushroom (*Pleurotus ostreatus*). Oyster mushrooms (*Pleurotus ostreatus*) are comparatively easy to cultivate so we chose them for project. The oyster mushroom has a broad, fan or oyster shaped cap spanning 5-25 cm; natural specimens range from white to gray or tan to dark brown; the margin is enrolled when young and is smooth and often somewhat lobed or wavy. The flesh is white, firm, and varies in thickness due to stipe arrangement. The gills of the mushroom are white to cream and descend on the stalk if present. Mushrooms have been attracting attention of mankind since ancient times and use of mushroom as food is as old as human civilization. They have considerable importance in the human diet as they are rich in protein, nonstarchy carbohydrates, dietary fiber, minerals, and vitamin B and have no cholesterol, and negligible amount of fat. Mushroom proteins are of high quality and they contain an abundance of essential amino acids [1]. Oyster mushroom is capable of handling the problem of protein malnutrition in developing and underdeveloped countries [2].

Aim of this study is to compare various parameters of oyster mushroom cultivated on different

substrate. Mushroom is being cultivated by cultivators on large scale as a side business and also consumed by lot of people and thus estimating the dietary components of it is necessary. Generally only paddy is used for cultivation of mushroom, but different substrates can also be used in combination of paddy for enhancing the quality of mushroom as we used sugarcane bagasses and paper, but it should be a waste material, so that it would be cost effective for cultivators to use it.

Scientific Name: *Pleurotus ostreatus*

RANK	Scientific Name & (Common Name)
Kingdom	Fungi
Division	Basidiomycota
Order	Agaricales
Family	Pleurotaceae
Genus	<i>Pleurotus</i>
Species	<i>P. ostreatus</i>

2. MATERIALS AND METHODS

2.1.Cultivation of Mushrooms

Material: Paddy, Autoclavable bags, Substrates-paddy, sugarcane molasses, Oyster mushroom spawns, Autoclave, Wooden box for cultivation, Aluminum foil, Spray bottle

Chemicals: Methanol, Formalin, Formaldehyde and potassium permanganate for fumigation, Water



Figure 1: Hanged Mushroom Bag



Figure 2: Bag Removal

Procedure: Wooden box was cleaned and fumigated for 24hr with formaldehyde and potassium permanganate. Paddy was soaked in hot water overnight. Paddy was autoclaved using autoclavable bags and sugarcane molasses and paper pieces were also autoclaved using aluminum foil. (121 degree Celsius, 15psi for 20 min) Next day- Bags were wiped with 1:1 formalin. Three bags were prepared, one with only paddy, other with paddy and paper pieces and last one with paddy and sugarcane molasses. During the preparation, base layer was of substrate, onto which spawns were added more at the periphery and less in middle portion. 5-6 such layers were prepared. Then all the bags were hanged in wooden box and sides of the box were sprayed with water for moisture. Daily we sprayed water on the sides of the wooden box. After 3-4 days bags were punctured using needle for exchange of gases.



Figure 3: Full Mushroom Growth

Mycelial growth was observed:

Paddy: on 10th day after hanging

Paddy and paper: on 6th day after hanging

Paddy and sugarcane: on 13th day after hanging

For each bag, when maximum mycelial growth was observed, bags were removed and the stacks were placed as it is in the wooden box for further growth.

Plucking of mushrooms:

Paddy: 33rd day after hanging

Paddy and sugarcane: 33rd day after hanging

Paddy and paper: 25th day after hanging

Observations during Cultivation: Paper + paddy bag mushrooms were grown more rapidly than other two bags mushrooms.

2.2. Analysis of Mushroom

Preparation of Mushroom Extract for Antioxidant Activity

SUBSTRATES	RESIDUE WEIGHT	D/W (ml)
PADDY	0.5g	25
PADDY + SUGARCANE	0.3g	15
PADDY + PAPER	0.78g	39

Mushrooms from each bag were plucked and air dried and fine powder was prepared. Extract of mushrooms were prepared for each bag. For preparation of extract, 5g powder of mushroom powder was boiled in 250 ml distilled water for 30 min. Then the solutions for each bag were centrifuged at 10,000 rpm for 15 min. Then supernatant was collected and filtered using what Mann filter paper. Then supernatants for each bag were taken separately in 3 beakers. Beakers were kept on sand bath till the dry residues were obtained. Those residues were weighed and according to initial concentration, distilled water was added.

Now the concentration for all 3 samples-0.02g per ml. Now these extracts were used for analysis of phenolic compounds

PHENOLIC COMPOUNDS

Estimation of phenolic compound from mushroom extract was done by using Folin-Ciocalteu reagent [3] with some changes. 1ml sample extract was diluted with 46 ml distilled water and 1 ml folin-ciocalteu reagent and mixed it for 3min. then 3ml of 2%v/v sodium carbonate was added and mixture was allowed to stand for 90 min at RT with intermittent shaking .Absorbance was calculated at 630 nm.

FLAVONOIDS CONTENT

The principle involved in Aluminum chloride ($AlCl_3$) colorimetric method is that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids [4] .Estimation of flavonoid content was done by methodology given by Chang et al[5] 1 ml extract was diluted with 4.3 ml of 80% (v/v) aq. Ethanol containing 0.1ml of 10% (v/v) aluminum chloride and o.1 ml of 1M aq. Potassium acetate. The mixtures were allowed to stand at RT for 40min and absorbance was measured at 415 nm.

ANTIOXIDANT ACTIVITY BY DPPH METHOD

DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. The color changes from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. Radical scavenging activity increased with increasing percentage of the free radical inhibition. The degree of discoloration indicates the free radical scavenging potentials of the sample/antioxidant by their hydrogen donating ability. The electrons become paired off and solution loses colour stoichiometrically depending on the number

of electrons taken up 9 [6].

Brand-Williams et al [7]. The solution of 0.1mM DPPH was freshly prepared such that its absorbance lied between 2.4 to 2.8 at 517 nm. Negative control was set up by taking 500 microliter of freshly prepared DPPH and 500microlitre of methanol. Range of concentration of mushroom extract was prepared. (0.5 mg/mL, 1mg/mL, 2mg/mL, 5 mg/mL) by considering the original extract as 100%. For preparation of blank, 500 microliters from these samples was transferred to another eppendorf and 500 microliter of methanol was added to the stock solutions. To the 500 microliter of the sample from each tube that was transferred to the eppendorf, 500 microliter of DPPH was added. This was taken as test sample. All this was carried out in dark as DPPH is light sensitive. All the eppendorfs containing test samples, blanks and negative control were kept in dark for half an hour. The readings were taken using spectrophotometer at 517 nm using the amber coloured cuvette. First the reading of negative control was taken and then of blank and test in the increasing concentrations. Absorbance at 517nm.

Negative control (NC): 2.5744.

% scavenging activity = $\frac{NC - (Test - Blank)}{NC} \times 100$



Figure 4: DPPH ASSAY

PREPARATION OF MUSHROOM EXTRACT FOR CARBOHYDRATES

Mushrooms from all three bags were collected separately. Then these mushrooms were air dried and fine powdered was prepared from that mushrooms. In borosil beaker 5 gram of mushroom powder of all three samples was added to 50 ml of distilled water. These beakers were kept in boiling water bath for 3 and half hour. These solutions were centrifuged at 7000 rpm for 15 minutes. Supernatant was collected and filtered with what Mann filter paper. Obtained filtrates were used for further analysis.

CARBOHYDRATES

Analysis of carbohydrates was carried out by methodology given by Dubois *et al* [8] with some changes. 1ml extract was diluted with 1ml phenol and 5ml 96% sulphuric acid and it was shake

well. The mixture was kept in boiling water bath for 20 min. absorbance was taken at 500nm.

PROTEINS

The principle involved in Lowry method is under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. Estimation of proteins was done by using Lowry's method [9] with some changes. 1ml extract was diluted with 0.5 ml sodium hydroxide and 5 ml of alkaline copper reagent. The mixture was incubated at RT for 30 min. Then 5 ml of folin- ciocalteu reagent was added to it after that it was incubated at RT for 10 min. and absorbance was calculated at 660 nm.

3. RESULTS AND DISCUSSION

As absorbance is directly proportional to concentration we have only determined absorbance as the main aim of this study is comparison of dietary parameters of oyster mushroom grown on different substrates.

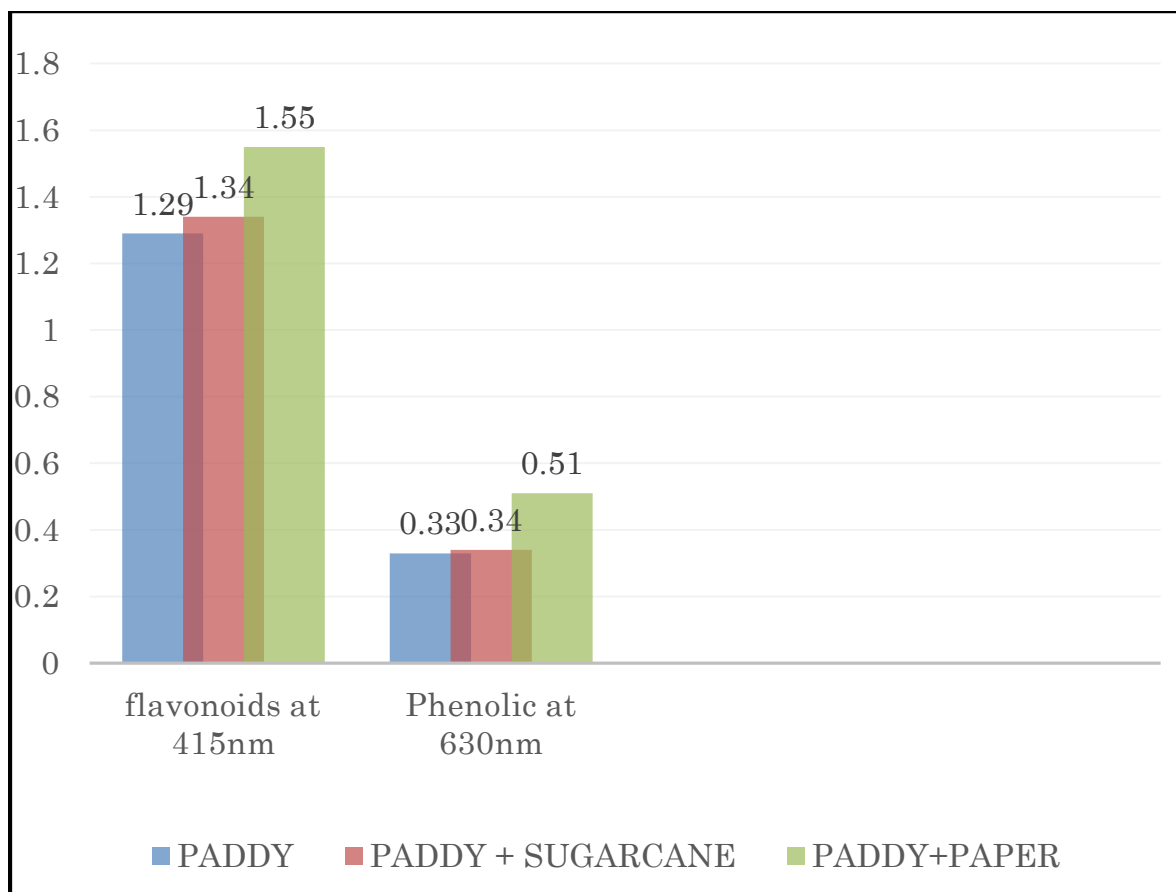


Figure 5: Analysis flavonoids and phenolic content

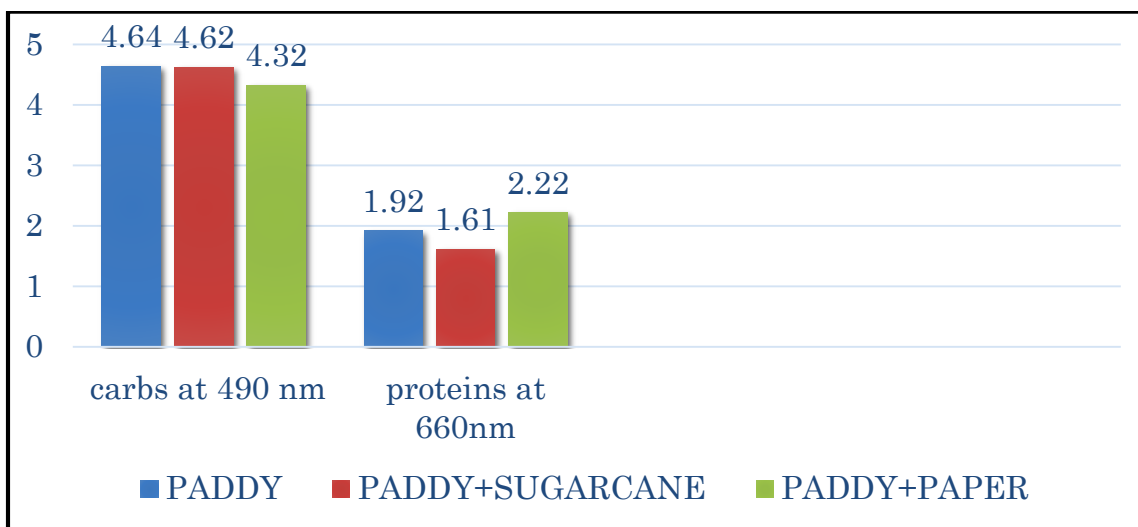


Figure 6: Analysis of carbohydrates and proteins

From fig 5-6, we can see that mushroom grown on paddy+ paper substrate have high proteins, flavonoids, phenolic content and antioxidant activity than the other mushrooms grown on only paddy and paddy+sugarcane bagasses. But at same time mushrooms grown on paddy+paper substrate show low carbohydrate content than the other than two. Further chromatographic study can be done on used mushroom sample for determining potential components present in sample giving these results. Other substrates like waste carrot peels, beetroot peels, tea powder, saw dust, etc. and many such organic waste can also be used.

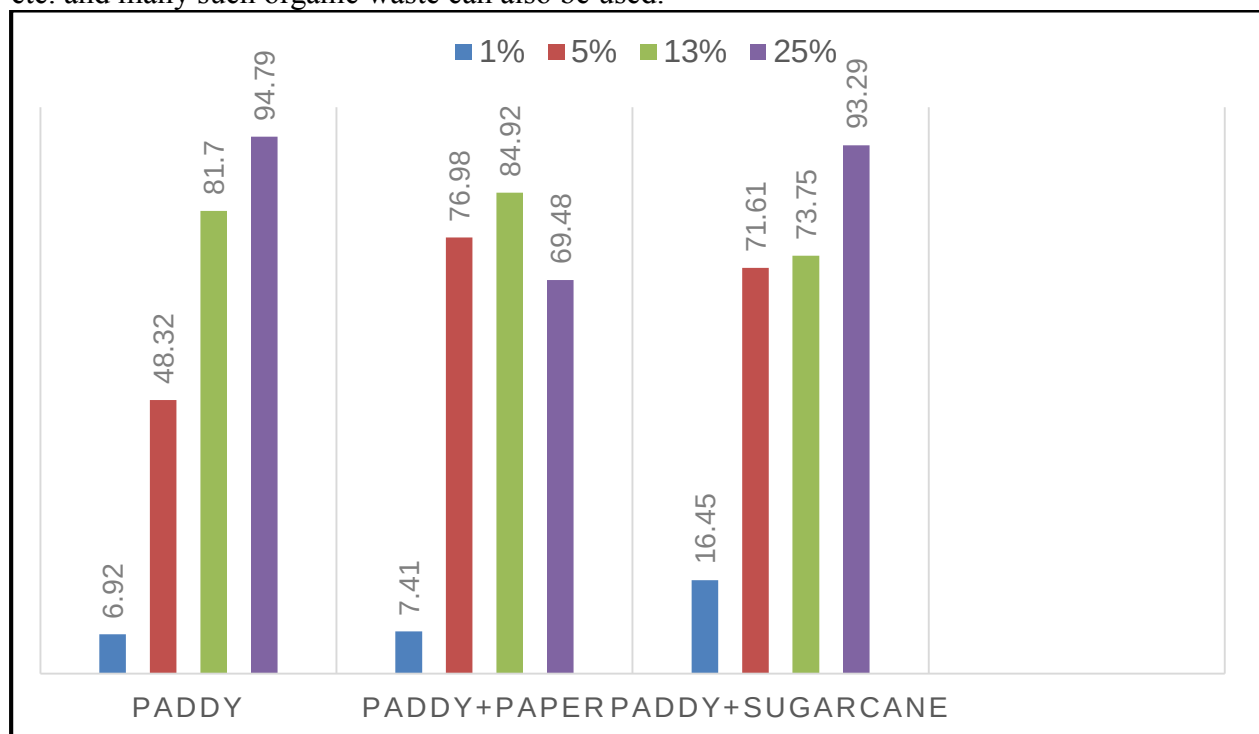


Figure 7: Antioxidant activity by DPPH method

4. CONCLUSION

Through this study we can conclude that paddy and paper is better substrate for high content of proteins, flavonoids, phenolic compounds and also for high antioxidant activity. Paddy with paper is also better substrate to lower the carbohydrate content.

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

We have no conflict of interest.

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Formulation of Centre Filled Probiotic Yogurt Hard Candy

P. MULE, DR. S. SONAWDEKAR*

School of Biotechnology and Bioinformatics,
D. Y. Patil Deemed to be University, Navi Mumbai, India.

ABSTRACT: Probiotic food products have taken over the food industry in recent years. Beneficial effects of probiotics including intestinal health and improvement in immune response have contributed to increase in interest in research work done on it. Yogurt with probiotics has been considered as an ideal food traditionally. Also hard candies are also been consumed traditionally. The main objective of this study is to formulate a hard candy which is center filled with probiotic yogurt. Probiotics strains used in present study are *Lactobacillus acidophilus* and *Streptococcus thermophilus*. In this study two type of center filling were made one which is semi dried form and another one is completely dried powdered form. In this study variations in concentration of glucose syrup were tried of which 20% by weight of sugar was found to be compatible. Sensory evaluation was carried out which stated that candy with semi dried center filling was more acceptable than that of candy with powdered center filling.

KEYWORDS: Probiotics, yogurt, hard candy, center filled candy

***Corresponding Author:** Dr. Sheetal Sonawdekar
School of Biotechnology and Bioinformatics,
D. Y. Patil Deemed to be University, Navi Mumbai, India.
Email Address: Sheetal.sonawdekar@dypatil.edu

1. INTRODUCTION

Yogurt

Yogurt has long been known in human history as a way of preserving milk. Yogurt is defined as “fermented milk where the fermentation has been carried out with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* with or without lactic acid producing microorganisms where they have to be viable during shelf-life at pH 4.5 with minimum protein content (measured as crude protein) of 30 g/kg” [1]. Despite the distinctive acidity of natural yogurt, the consumption of yogurt or other cultured milk products is believed to have additional health promoting benefits to the host [2]. Since then, production of cultured milk products has become commercially important worldwide. Today, the technology of yogurt-making has become more advanced, which delivers more functional health benefits (e.g. probiotics) as well as application of strict hygiene control (e.g. HACCP and GMP) along with a variety of yogurt types to suit individual tastes (e.g. low fat, reduced sugar) [3].

Probiotics

The term was originally coined to describe substances produced by one microorganism that stimulated the growth of others and was later used to describe tissue extracts that stimulated

microbial growth and animal feed supplements exerting a beneficial effect on animals by contributing to their intestinal flora balance [4]. The internationally endorsed definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. As far as nutrition is concerned only the strains classified as lactic acid bacteria (LAB) are of significance and among them the ones with the most important properties in an applied context are those belonging to the genera *Lactococcus* and *Bifidobacterium* [5]. Two other species playing an important role in the dairy industry, although not strictly considered as probiotics are *Streptococcus thermophilus* and *Lactococcus lactis*, two of the most commercially important lactic acid bacteria [6]. Some of the most important representatives are listed in table 1.

<i>Lactobacillus</i> species	<i>Bifidobacterium</i> species
<i>L. acidophilus</i> <i>L. casei</i> <i>L. crispatus</i> <i>L. gallinarum</i> <i>L. gasseri</i> <i>L. johnsonii</i> <i>L. paracasei</i> <i>L. plantarum</i> <i>L. reuteri</i> <i>L. rhamnosus</i>	<i>B. adolescentis</i> <i>B. animalis</i> <i>B. bifidum</i> <i>B. breve</i> <i>B. infantis</i> <i>B. lactis</i> <i>B. longum</i>

Table 1: Microorganisms considered as probiotics [7]

Hard candy

The word “candy” comes from Arabic qandi, derived from Persian qand, meaning “sugar”. It comes in many wonderful flavors including milk chocolate, caramel, peppermint, dark chocolate, butter scotch and various other fruit and mint flavors. It also comes in many consistencies such as chewing gum, hard candy, soft candy and all sorts of great delicious configurations and shapes [8].

2. MATERIALS AND METHODS

2.1. Materials

For yogurt preparation, homogenized, pasteurized, toned milk with fat 3%, 8.5 solid not fat content (SNF) and 3 g protein content was used. Yogurt starter culture named YOGURTMET was used which contained live active bacterial culture of *L. bulgaricus*, *L. acidophilus* and *S. thermophilus*. This starter culture also contained milk powder and sucrose. For formulation of candy, Glucose syrup (GS), table sugar, oil based guava flavor, rose pink food color were obtained from local market. For the center filling, Red chili powder, chaat masala, salt and sugar were used in the filling and were obtained from local market.

2.2.Methods

Yogurt preparation

For yogurt preparation 1 liter of toned milk was used. It was boiled at 80°C and was cooled to 45°C. To this cooled milk starter culture was added. It was allowed to incubate at 45°C for 4-5 hours. To terminate the fermentation it was refrigerated.

Preparation of central filling

Hung curd was prepared from the yogurt obtained by above procedure. The whey was drained from the yogurt. Approximately 250-260 gm of hung curd was obtained after hanging it for 7 hours at room temperature. This obtained hung curd was mixed with 15.62% sugar, 0.6% salt, 0.4% red chili powder, and 0.4% chat masala. This mixture was blended with the help of hand blender to obtain smooth consistency. To make the centre filling in semi dried form it was dried in tray dryer at 50°C for 6 hours in stainless steel plates. After the desired consistency was obtained it was scrapped off and stored in refrigerator at 4°C.

Procedure for making candy

40 gm sugar, 8 gm GS and 17 ml water was measured and kept on medium heat in a stainless steel vessel. This mixture was allowed boiled. With the help of cooking thermometer temperature was monitored. After the temperature reaches 146°C the burner was switched off. At this stage flavoring and coloring agents were added as per Good manufacturing practices (GMP). After mixing the flavor and color the candy mixture is poured into mould and the center filling of yogurt was inserted. The mixture was allowed to set and take the shape of mould. The final product was then wrapped into transparent plastic wrappers and stored into air tight container.

Estimation of TPC

Total plate count (TPC) for viable microbial count of probiotics in yogurt was outsourced from Varni analytical laboratory. Method followed was IS-5402:2012.

Sensory evaluation

Sensory evaluation was done for the final product for the parameters such as appearance, aroma, colour, flavor, taste, after taste and overall acceptability.

Sensory evaluation of candy was carried out by 11 penalist. The ratings for parameters mentioned above were based on the nine point hedonic scale [9].

9 = Like extremely

8 = Like very much

7 = Like moderately

6 = Like slightly

5 = Neither like or dislike

4 = Dislike slightly

3 = Dislike moderately

2 = Dislike very much

1= Dislike extremely

Proximate analysis

Proximate analysis of the final product was outsourced from Varni analytical laboratory. The parameters tested were moisture, total ash, protein, fat, carbohydrates, and energy. Methodology used for determining moisture, total ash, protein, fat was followed according to FSSAI MANUAL-4.

3. RESULTS AND DISCUSSION

3.1.Assessment of different parameters of yogurt and hung curd

Sample	Fat (%)	Titrate acidity (%)	pH
Yogurt	3	0.9	4.5
Hung curd	33	2.4	4.4

Table 2: Assessment of different parameters of yogurt and hung curd

3.2.Total weight of hung curd

550 gm of hung curd was obtained from 1 kg of yogurt. While making semi dried form of filling of this 550 gm, about 400 gm of final product was retained. And while making powdered form of filling from 550 gm of hung curd 278 gm of final product was obtained.

3.3.Estimation of TPC

The TPC for viable count was found to be 1.7×10^3 cfu per gram of the product.

This count doesn't match the specification set by FSSAI regulation for probiotic food. Therefore additional edible probiotic culture was added to match the regulations and TPC was conducted again. The result obtained from second TPC was 1×10^7 cfu per gram of the product.

3.4.Proximate analysis of final product

Sr no.	Tests	Results (per 100g)	Unit
1	Moisture	4.01	G
2	Total ash	1.45	G
3	Protein	5.71	G
4	Fat	1.91	G
5	Carbohydrates	86.93	G
6	Energy	387.70	kCal

Table 3: Proximate analysis

3.5.Sensory evaluation

Sensory evaluation was done for both the samples on 9 point hedonic scale. Parameters considered for the evaluation were appearance, aroma, color, flavor, taste, after taste. The average rating for each parameter of final product are mentioned in the table below.

Parameter	Sample
Appearance	8
Aroma	7
Color	7
Flavor	8
Taste	8
After taste	8
Overall acceptance	8

Table 4: Average ratings of all parameters of sensory evaluation

4. DISCUSSION

According to the Codex Alimentarius Commission yogurt should have a maximum fat content of 15%. In order to meet this, the FAO/WHO standards specify that milk should be standardized with the minimum SNF and milk fat content of 8.2% and 3% respectively for yogurt manufacture. The milk used to set yogurt in this study was as per the above mentioned specification. The fat content of yogurt prepared was found to be 3%.

Hard candy centre filled with probiotic yogurt was prepared. To determine the exact temperature required for candy making various methods are available which are either by candy thermometer or by cold water test. Method in which candy thermometer is used was followed here because this gives the exact temperature needed to formulate hard candy. Flavor used in the preparation was oil based because the temperature needed for candy formulation is high. These flavors can tolerate high temperature and do not lose their properties. On the other hand water based flavor do not survive high temperature and lose the properties. Optimum average weight of candy should be in between 4 to 7 gm [8] according to Reena Hoonda stated in their study in 2015 about formulation of herbal candies. Candy formulated in the present study weighed about 5 gm.

It is usually observed that candy grains during storage. It changes from clear to opaque in appearance and tends to lose flavor. Graining during storage is usually accompanied by sticyness. It also progresses from the surface towards center occasionally, graining will occur throughout the candy equally and candy will remain dry. When hard candy batch grains during production it loses its plastic consistency and becomes short in texture, its appearance also changes from clear to opaque. The candy formulated in present study also began to grain after a week of its formulation. After reviewing the causes it can be concluded that the storage conditions were harming the candy resulting in graining, loss of texture and loss of clarity.

Various types of evaluations were done to check the quality of the product. Sensory evaluation including parameters like appearance, aroma, color, flavor, taste, overall acceptability was done by 11 panelist members.

The product was tested for TPC to obtain viable microbial count of probiotics in yogurt. After all the process done to obtain final product the TPC found was matching the FSSAI regulations for probiotic food.

The candy was subjected to physicochemical analysis to analyze for moisture, total ash, protein, fat, energy and carbohydrate content. The results obtained were in the range stated by specifications of FSSAI regulations for beverages and confectionary.

Overall this is a suitable formulation for the delivery of probiotics with has number of advantages such as good in taste, easy to carry because small in size, easy to store, effective.

5. CONCLUSION

Different concentration of GS were tried to formulate the hard candy. By using 20% GS the formulated product was least sticky and hence palatable. The chilly powder, sugar and salt used in the yogurt was of suitable concentration of all other concentration tried and gave great acceptance. Initially present viable count after all the processing was done in yogurt was found to be less than required. Hence addition of edible probiotic culture was done which gave satisfactory viable count.

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7. CONFLICT OF INTEREST

There is no conflict of interest between the two authors.

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Histamine Reduction by Ultrasound Treatment and Histamine Measurement by Modified Calorimetric Methods

S. SAHASRABUDHE, A. TIWARI, J. AICH, S. KUDALE, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India

ABSTRACT: Histamine is a biogenic amine compound. It plays an important role in local immune responses and regulating physiological function. Histamine is a compound found in numerous foods and beverages, which causes food allergy. The reduction of histamine from fermented food is a challenging task for fermented food processing industry. Ultrasound treatment at 50Hz, Ultraviolet rays and synergistic effect of ultrasound and ultraviolet ray exposure was given to beer samples for 1 hour, 2 hour, 3 hour, 4 hour and 5 hour. The hourly basis samples were analyzed for histamine content by the modified colorimetric determination method. Color change was analyzed based on L, a, b values. Sensory analysis was carried out by 9 points hedonic scale from 30 untrained panelists. The ultrasound treatment, ultraviolet treatment and synergistic ultrasound-ultraviolet treatment for 5 hours to beer reduced histamine level by $28.82 \pm 1.36 \%$, $41.04 \pm 1.14 \%$, $50.81 \pm 1.30 \%$ respectively without any rejection of sample from a sensory panelist without any color change in beer samples.

KEYWORDS: Beer, Histamine, Food Allergy, Ultrasound, Ultraviolet, synergistic ultrasound-ultraviolet

***Corresponding Author: Mr. Ashish Dabade**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Food Biogenic amines are generally considered as food allergens synthesized in the microbial metabolic process [1]. The biogenic amines are considered as a microbial fermented byproduct. Until now, relatively less work has been carried out on biogenic amines. Decarboxylation of amino acids results into the synthesis of biogenic amines. Histidine, Tyrosine, Tryptophan, and Lysine convert to Histamine, Tyramine, Tryptamine and cadaverine respectively. The decarboxylation process acts as a catalyst for microbial spoilage as well as food allergen synthesis. The biogenic amines determine the shelf life of food on the basis of biogenic amine content. Monoamine oxidases and diamine oxidases degrade the biogenic amines. Histamine and tyramine are a major concern from food safety point. The accepted maximum limit of histamine, tyramine and phenylethylamine should be 100mg, 800mg and 30mg. The main biogenic amines synthesis is caused by Enterobacteriaceae, lactobacilli, pediococci, and enterococci group. Biogenic amine production synthesis rate increases with concentration of proteolytic enzyme at stationary phase. The redox potential also affects the biogenic amine production [2]. The reduction of biogenic amines was tried

to be carried out by methods of preservation but feasibility for industrial scale is a major concern [3]. The physical methods like UV treatment also proved that up to 85% biogenic amines can be reduce by UV light [4]. The biogenic amines such as histamine can easily detected by colorimetric method [5]. The histamine level in beer can reach up to 100 to 300 µg/liter depending on the processing.[6].

2. MATERIALS AND METHODS

2.1.Materials

Beer (Bira 91 strong) a top fermenting Pale ale and a “High-Intensity Wheat Beer”, with an alcohol content of 7% ABV purchased from local market.

Chemicals and Reagents

Histaminedihydrochloride (HPLC grade), Sulfanilic acid, hydrochloric acid Sodium nitrite and Sodium Carbonate (Analytical grade) procured from SRL laboratories. Activated carbon (particle size-300 mesh) procured from S.D.Fine chemicals limited)

2.2.Methods

The study was approved by ethics committee of Dr. D.Y Patil University, Also written informed consent was obtained by all individual panelists prior to the tasting.

Ultrasound Treatment

5 glass beakers of beer sample of volume 100 ml was kept in ultrasonication chamber (50Hz) (Make-Dakshin). Each beaker of beer sample was withdrawn from the chamber with an interval of 1 hour. After 5 hours, 6 samples were collected including control sample.

Ultraviolet treatment

5 glass beakers of Beer sample of volume 100 ml was exposed to ultraviolet light to fixed ultraviolet wavelength 395 nm (Make- amiciKart). Each beaker of beer sample was withdrawn from exposure chamber with 1-hour interval. After 5 hours, 6 samples were collected including control sample.

Synergistic treatment with ultrasound and ultraviolet

5 glass beakers of beer sample of volume 100 ml each exposed to fixed ultraviolet wavelength of 395 nm. Each beaker beer sample was withdrawn from UV exposure chamber with 1-hour interval. Each UV treated withdrawn sample further kept in ultrasound chamber. The ultrasound exposure time was same as UV chamber time. After 5 hours, 6 samples were collected including control sample.

Histamine Analysis by calorimeter

The histamine content was analyzed of each sample by calorimetric methods at 496 nm as mentioned in [5]. The method was modified to handle liquid samples. The sample decolonization

was carried out with activated carbon. The colorless samples were analyzed by calorimetric method.

Color Analysis

The samples were also analyzed for its color change properties color analysis software (Research Lab). The ΔE value calculated by the formula:

$$\Delta E = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2}$$

Sensory Analysis

The beer samples treated with ultrasound, ultraviolet and synergistically were analyzed for its sensory attributes like color, aroma mouth feel, flavor, appeal, overall acceptability. An untrained panel was set-up to analyze the above attributes, each member of the panel was asked to evaluate the samples with affective testing along with the rating it with the reference to the hedonic scale from 0-9.

Statistical and comparative analysis

One-Way ANOVA compare means were analyzed with software SPSS. The significant difference study was carried out with homogeneous subset comparison by Duncan method.

The reduction of histamine level calculated by formula:

Reduction in percentage =

$$(\text{Control Histamine Level} - \text{After treatment Histamine Level}) \times 100 / (\text{Control Histamine Level})$$

3. RESULTS AND DISCUSSION

3.1.Determination of Total Phenolic Content

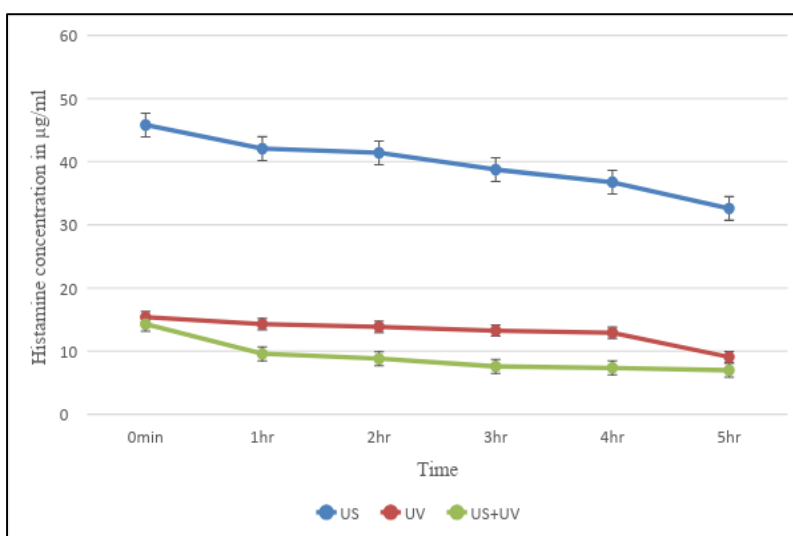


Figure 1: Treatment comparison Histamine levels in beer with ultrasound, ultraviolet and synergistically treated samples. US- Ultrasound, UV- Ultraviolet, UV+US- Synergistic. ($n=3$).

Uses Harmonic Mean Sample Size = 3.000 and Subset for alpha = 0.05

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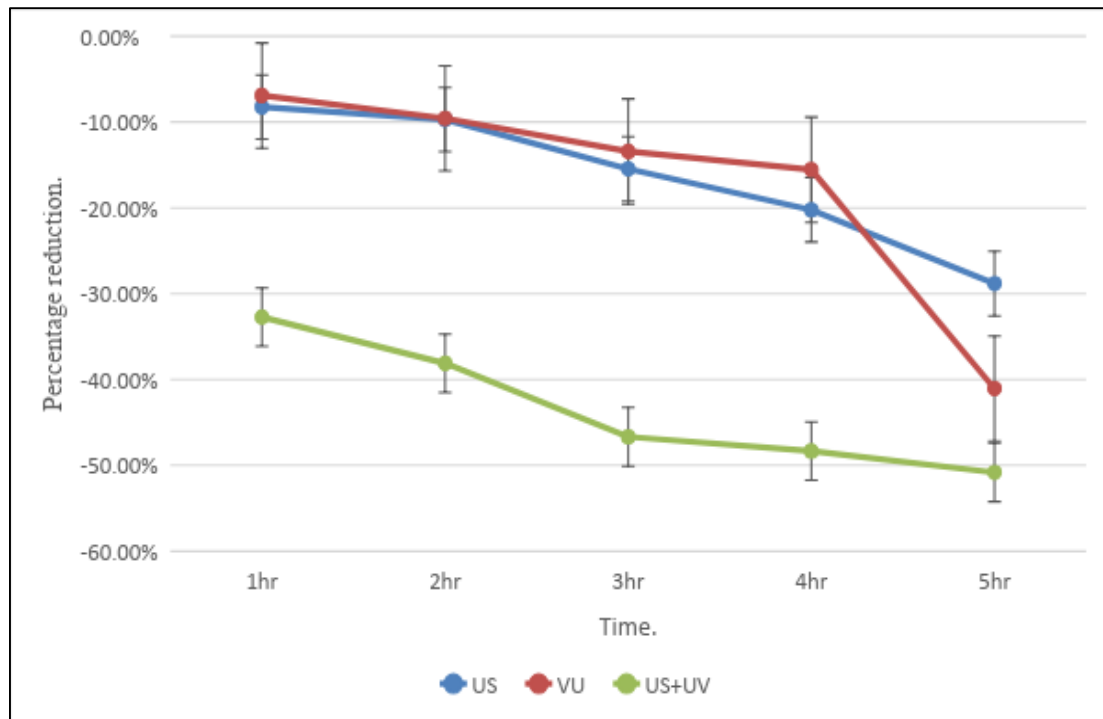


Figure 2: Comparison of percentage reduction in histamine level in beer with ultrasound, ultraviolet, and synergistic treatment. US- Ultrasound, UV- Ultraviolet, UV+US- Synergistic ($n=3$)

Uses Harmonic Mean Sample Size = 3.000 and Subset for alpha = 0.05

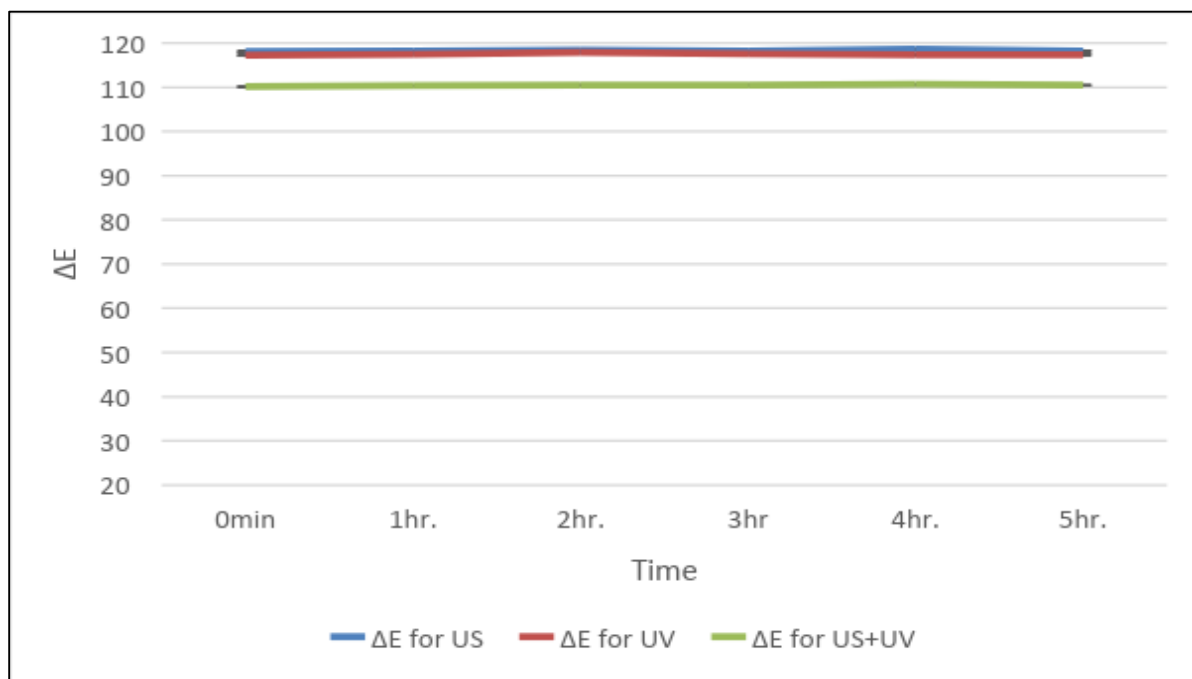


Figure 3: ΔE based color comparison of beer samples in ultrasound, ultraviolet and synergistic treatment. US- Ultrasound, UV- Ultraviolet, UV+US- Synergistic. ($n=3$)

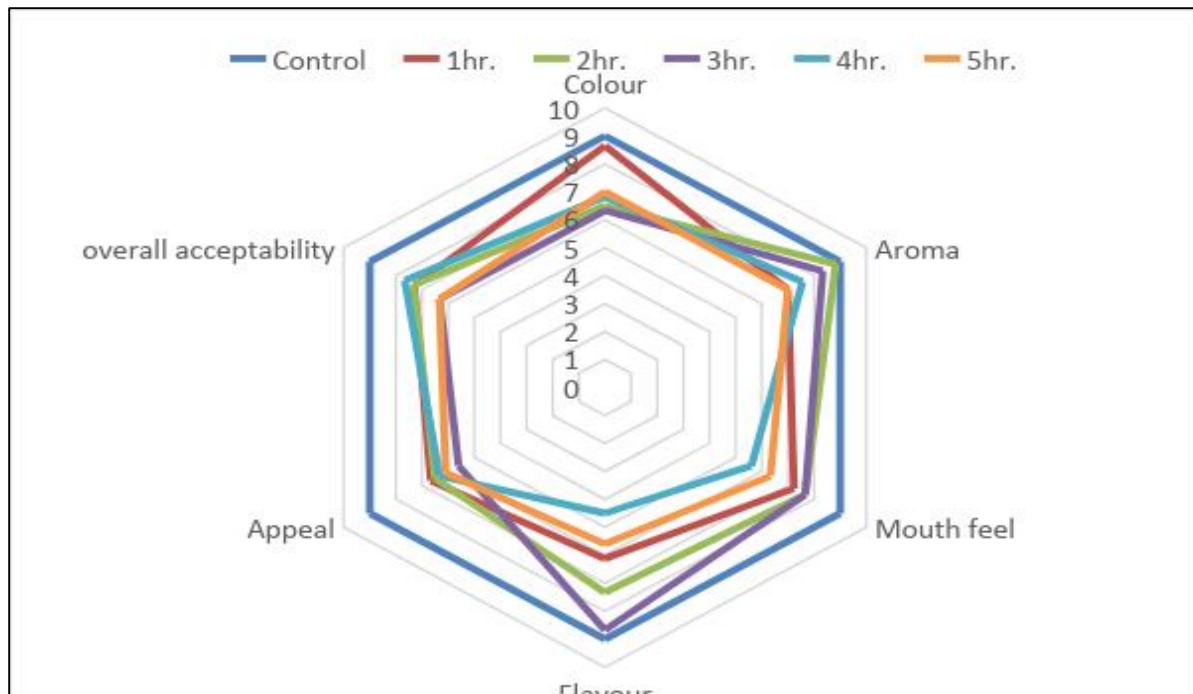


Figure 4: Beer Sensory analysis Post Ultrasound treatment. ($n=30$)

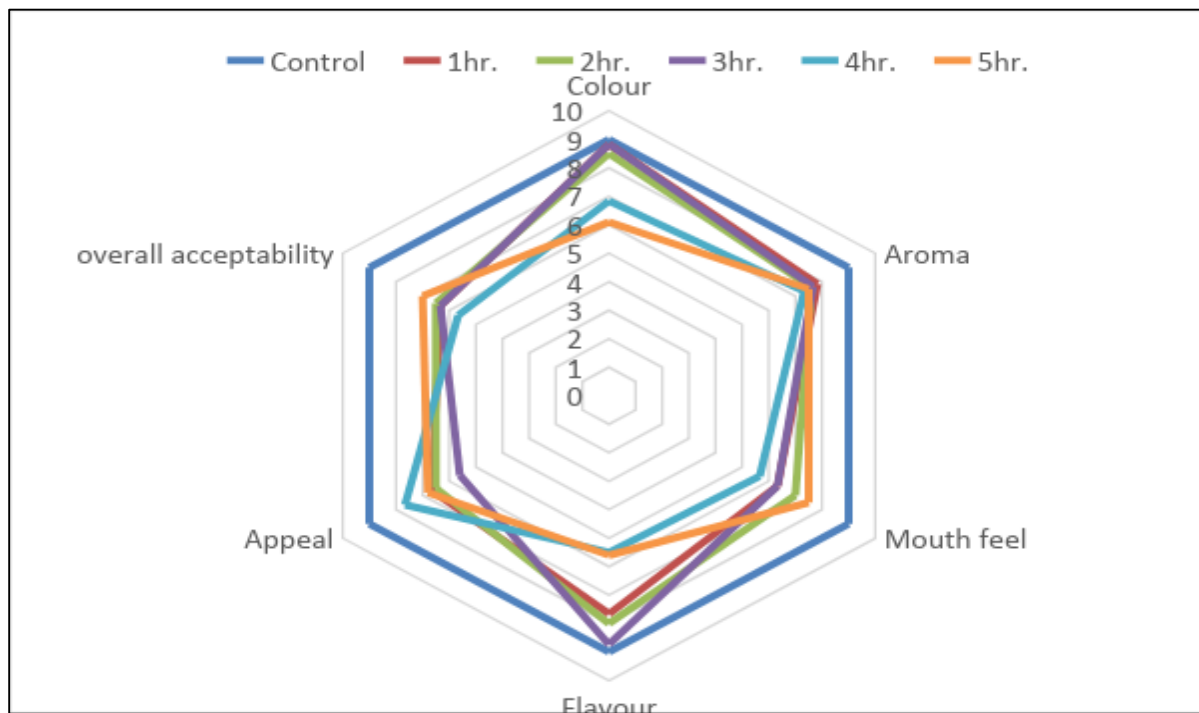


Figure 5: Beer Sensory analysis Post Ultraviolet treatment. ($n=30$)

Ultrasound Treatment

The 5-hour exposure of ultrasound treatment was showing significant reduction $32.60 \pm 0.13 \mu\text{g/ml}$

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of histamine levels than control sample $45.15 \pm 0.18 \mu\text{g/ml}$ (Fig.1.) The mean difference was significant at the 0.05 level for samples for 3 hour, 4 hour and 5 hour as compared to control sample. The 3 hour, 4 hour and 5 hour histamine levels are significantly not different to each other (Table.1) Hence, 3-hour exposure to 50 Hz. Ultrasound treatment can reduce the histamine level-up to $15.46 \pm 5.25\%$ significantly.

Time	Histamine $\mu\text{g/ml}$ (US)	Histamine $\mu\text{g/ml}$ (UV)	Histamine $\mu\text{g/ml}$ synergistic (UV & US)
Control	45.15 ± 0.18^c	15.39 ± 1.13^c	14.28 ± 0.09^c
1hr.	42.07 ± 3.6^b	14.62 ± 0.39^b	9.58 ± 0.94^b
2hr.	41.40 ± 6.86^b	13.86 ± 0.20^b	8.81 ± 0.63^c
3hr	38.75 ± 3.06^a	13.26 ± 0.28^a	7.59 ± 0.27^a
4hr.	36.53 ± 3.64^a	12.92 ± 0.16^a	7.36 ± 0.64^a
5hr.	32.60 ± 0.13^a	9.08 ± 1.99^a	6.98 ± 0.14^a

Table 1: Histamine levels in beer with ultrasound, ultraviolet and synergistically treated samples. US- Ultrasound, UV- Ultraviolet, UV+US- Synergistic ($n=3$)

^a Uses Harmonic Mean Sample Size = 3.000 and Subset for alpha = 0.05

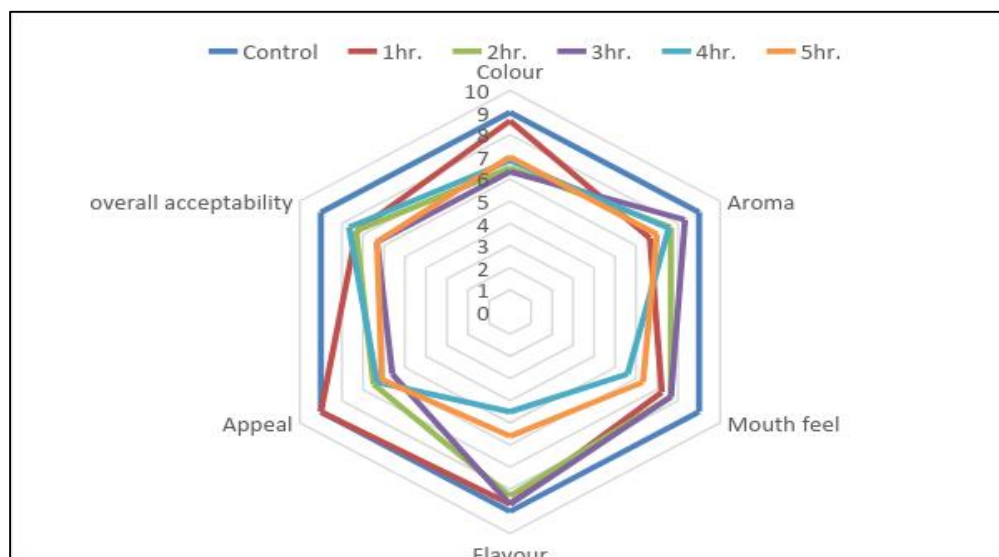


Figure 6: Beer Sensory analysis Post synergistic treatment. ($n=30$)

Ultraviolet treatment

The 5-hour exposure of ultraviolet was showing significant reduction $9.08 \pm 1.99 \mu\text{g/ml}$ of histamine levels than control sample $15.39 \pm 1.13 \mu\text{g/ml}$ (Fig.1.). The mean difference was significant at the 0.05 level for samples for 3 hours, 4 hours and 5 hours as compared to control sample. The 3 hour, 4 hour and 5-hour histamine levels are significantly not different to each other (Table.1). Hence, 3-hour exposure to 395nm wavelength ultraviolet treatment can reduce the

histamine level-up to $13.42 \pm 9.33\%$ significantly.

Synergistic treatment with ultrasound and ultraviolet

The 5-hour exposure of ultraviolet and ultrasound was showing significant reduction 6.98 ± 0.14 $\mu\text{g/ml}$ of histamine levels than control sample 14.28 ± 0.09 $\mu\text{g/ml}$ (Fig.1.). The mean difference was significant at the 0.05 level for samples for 3 hours, 4 hours and 5 hours as compared to control sample. The 3 hour, 4 hour and 5-hour histamine levels are significantly not different to each other (Table.1). Hence, a 3-hour exposure to 195 nm ultraviolet and 50 Hz. Ultrasound treatment can reduce the histamine level-up to $46.67 \pm 2.00\%$ synergistically.

Color analysis

Color analysis was not shown any significant difference in color ΔE value. The color was unaffected with any of the treatment (Fig.3).

Sensory Analysis

9 point hedonic scale spider chart (Fig.4) (Fig.5) (Fig.6) represents that all the samples treated with ultrasound were shown a slight change in sensory parameters due to de-carbonation of wine in 5 hour. The carbonation after post synergistic treatment can overcome objections of the panelist. The sensory panel accepted samples without any rejection.

4. CONCLUSION

The reduction in histamine by the physical treatments like ultrasound, ultraviolet and combination effect of the both was found positive. 3-hour exposure of synergistic treatment can be effective to reduce the histamine level from beer up to $46.67 \pm 2.00\%$. 3 hour can be considered as an optimized time for histamine reduction as compared to 4 and 5 hours based on statistical analysis study.

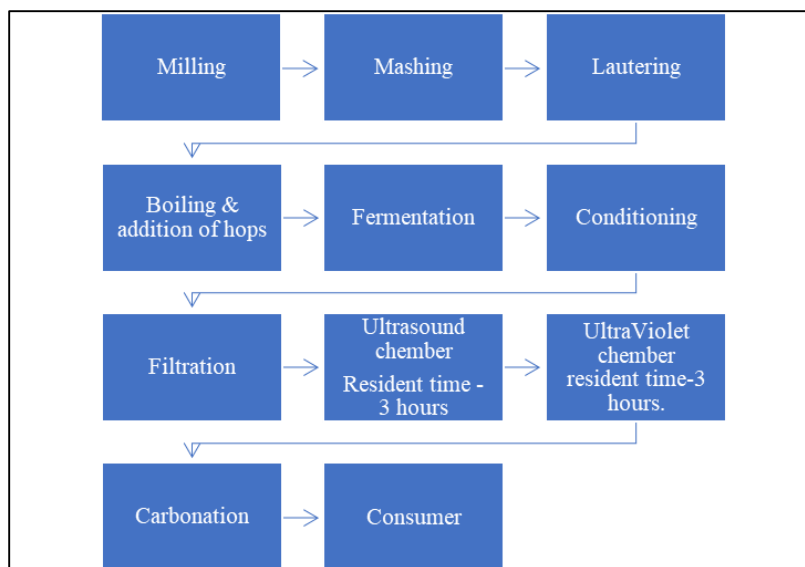


Figure 7: Modification in beer manufacturing process

Modification in beer manufacturing process can be used to reduce the histamine levels from beer (Fig.7). This new modification development will not result in less histamine beer but also it promises economical modification at current industry setup.

5. ACKNOWLEDGEMENT

I am thankful to Mr. Amar Srivastava & Mr. Anil Jacob at Brew crafts microbrewing pvt. Ltd. Pune (Doolally -Craft Beers and a cider) for providing us with beer samples and being a constant support in the process of project tenure.

6. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Acrylamide Reduction in French Fries by using Monosodium Glutamate

S. SALIM, K. BHOIR, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India

ABSTRACT: Acrylamide is the major concern in fried and baked foods. The major pathway leading to acrylamide formation is a part of the maillard reaction with asparagine and reducing sugars. The reduction of acrylamide was carried out by monosodium glutamate (MSG). The French fries sample were coated with 0.5%, 1%, 1.5%, and 2% MSG. The analysis of acrylamide was carried out by HPLC method. The acrylamide content in 0.5%, 1%, 1.5%, and 2% MSG coated French fries sample were found to be 0 ± 0 , 0.723 ± 0.626472 , 0.145333 ± 0.009504 , 0 ± 0 respectively. The acrylamide content in 0.5%, 1%, 1.5% and 2% coated French fries samples was found to be 100%, 91.01%, 98.193%, 100% in 0.5%, 1%, 1.5% and 2% respectively. No sample were rejected by the untrained panelists.

KEYWORDS: Monosodium Glutamate, Acrylamide, Maillard reaction, Edible coating, French fries.

*Corresponding Author: Mr. Ashish Dabade

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Potato based products are: French fries, chips and wafers, potato wine. Potato specialties are Dehydrated potato products, Frozen products, Potato starch, Potato powder, Flakes and pellets and Liquid glucose products [1]. French fries, finger chips or French-fried potatoes are baton net or allumette-cut deep fried potatoes. They are served hot, either soft or crispy, and generally eaten as a snack and commonly appear on the diner menus, fast food joints, bars and pubs. McCain rules with a market share of over 90%. Other leading brands, from Sumeru to Pagro to Al Kabeer, get their labelling done from either McCain or regional players such as Coimbatore-based Golden Fries and Agra-based BB Foods [2].

Frying is a widely used cooking method that creates unique textures and flavours in foods. Heating oil for frying at temperatures above 120°C can generate potentially cancer causing or carcinogenic compounds. Other carcinogenic compounds such as aldehydes, acrolein and acrylamide are also formed in fried or deep fried foods [3].

Health hazards of high fat food- The most primary health hazard is weight gain due to the excess amount of protein and carbohydrate intake for the body. The excess weight gain in turn may lead to slower metabolism and heart diseases formed due to the excess settlement of fat deposits in the arteries and around the heart. Constipation and irregular bowel movement can also be an outcome of high fat food consumption in the common man's regular diet. French fries come under the category of high fat foods as they contain relatively higher amounts of fats than raw potato tuber

[4].

High concentrations of acrylamide are found in fried potatoes and potato chips and, more generally, in starch-containing foods cooked at high temperatures. Long-term studies in rats and mice supported a dose–exposure relation between acrylamide and risk of cancer of the lung, mammary gland, thyroid, oral cavity, and intestinal and reproductive tract [5].

Water activity in food plays a major role in reducing acrylamide formation. Acrylamide forms in food only when the water activity is below 0.8, whereas the acrylamide formation is high at water activity of 0.4 and below. However, the removal of acrylamide from heated foods such as biscuits and potato chips increases with the increase of water activity [6].

Monosodium Glutamate (sodium glutamate), is the sodium salt of glutamic acid, one of the most naturally occurring non-essential amino acids. Found naturally in tomatoes, cheese and other foods [7].

MSG is used in the food industry as a flavor enhancer with an umami taste that intensifies the meaty, savory flavor of food, as naturally occurring glutamate does in foods such as stews and meat soups [8].

FDA has given MSG its generally recognized as safe (GRAS) designation [9].

2. MATERIALS AND METHODS

15kg non-fortified Sunflower oil gift sample was provided by AAK KAMANI PRIVATE LIMITED, Amul happy treats French fries was purchased from local market, Monosodium Glutamate purchased from SIGMA, N-Hexane purchased from MERCK, HPLC Water for chromatography purchased from MERCK, Carrez 1 – Potassium hexaferrocyanide and Carrez 2 – Zinc acetate purchased from SRL.

2.1.Optimization

Sensory attributes of French-fries fried at different time and temperature combinations were evaluated by a non-trained sensory panel of 22 members to obtain the optimum combination.

2.2.For Monosodium Glutamate coating

0.5g, 1g, 1.5g 2g Monosodium Glutamate in 50ml distilled water. The frozen French fries for test samples were each dipped for 10 seconds and then fried at the optimized condition. Control sample and test samples were prepared to find the acrylamide formed in normal French fries using the following method.

2.3.Preparation French fries samples

Prepared French fries samples were pulverized well, Add 5 grams of each samples was weighed in a centrifuge tube. In the next step, fat separation was performed using 7.5 ml of n-hexane, and then 17.5ml distilled water was added and centrifuged at 4000 rpm for 5 min. After then the upper aqueous phase was separated, and 1.25 ml of Carrez solution II (zinc acetate) were added to precipitate the soluble carbohydrate and protein. After separation this sample was thoroughly agitated, and then centrifuged for 5 min at 4000 rpm, and at last Supernatant was collected for

analysis.

2.4. Analysis by HPLC

The samples were filtered using a millipore filter with pore size 0.45µm and diameter 0.22µm. Sample analysis was done using high pressure liquid chromatography (HPLC) method. Sample analysis was done using water as the mobile phase at a flow rate of 1ml/minute. A C18 5µm column was used for analysis. Readings were compared with standard readings of known concentrations to obtain results to conclude if there has been any reduction in the formation of acrylamide or not [10].

2.5. Statistical analyses

The samples were carried out by SPSS Software module. The one way ANOVA, homogenised subset tests were carried out by statistical methods. The following formula was used to find % Reduction of Acrylamide.

$$\% \text{ Reduction of acrylamide} = \frac{\text{Untreated sample} - \text{Treated sample \%}}{\text{Untreated sample}}$$

3. RESULTS AND DISCUSSION

The sample without any monosodium glutamate content showed the acrylamide level upto 8.04 ± 0.01 ppm. The mono sodium glutamate treated samples showed significant reduction in acrylamide production. The 0.5%, 1.5%, 2%, 2.5% monosodium treated solution showed 0 ± 0 ppm, 0.72 ± 0.62 ppm, 0.14 ± 0.01 ppm, 0 ± 0 ppm respectively (Table.1).

There is a significant reduction ($p > 0.05$) of acrylamide when calculated using one way ANOVA. The homogeneous subset based statistical analysis proved that the application of 1%, 4% and 3% showed similar effect for acrylamide reduction by Duncan test. The 1% monosodium glutamate concentration is effective as 4% of monosodium glutamate treated samples. Minimum 1% MSG solution can effectively inhibit maximum acrylamide synthesis from fried product.

The reduction of acrylamide can be possible with 1% of monosodium glutamate upto 84.90%.

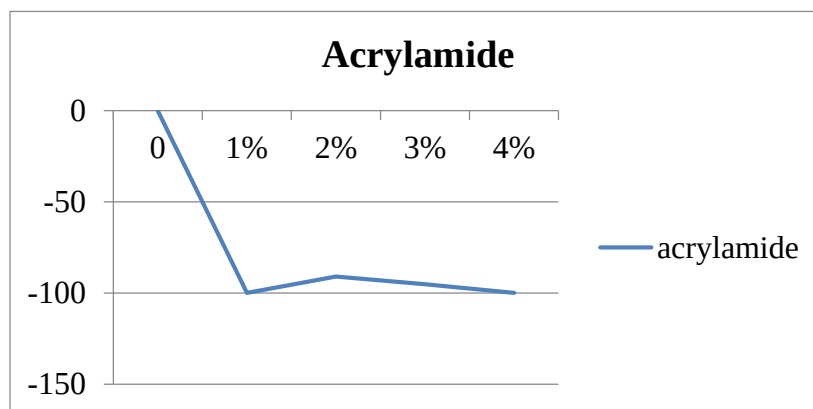


Figure 1: Percent reduction Acrylamide by MSG

Sensory attributes of French-fries fried at different time and temperature combinations were evaluated by 15 non-trained sensory panel of members to obtain the optimum combination which was found to be 175 degree Celsius for 3 minutes without any rejection.

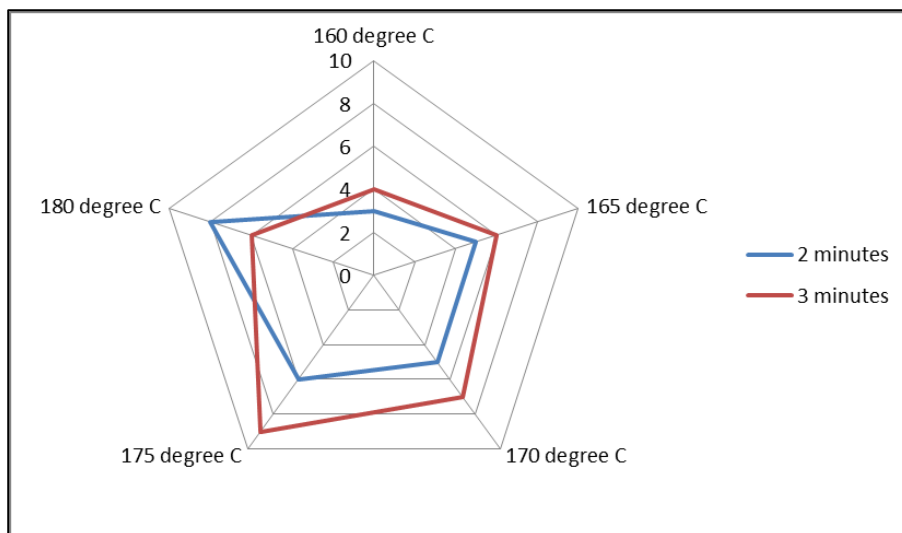


Figure 1.1: Sensory graph of optimization

4. CONCLUSION

Immersion of potato strips in MSG solution decreased the acrylamide formation after frying without reducing significantly their acceptability. Reduction of acrylamide in foods in the current scenario is an important study as it may help prevent activation of carcinogenic activity in humans which is a major problem the world is facing due to the modern lifestyle and eating habits. The above results represents that the application of 1% monosodium glutamate can reduce 84.90% of acrylamide from fried products significantly and cost effectively. The further application of taste enhancer E621 can be used in various fried product not only to improve to enhance taste of the product but also it can serve purpose to inhibit synthesis of carcinogenic products like acrylamide.

5. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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The Process Design for Reduction of Biogenic Amine from Fermented Wine

A. IYER, V. MISHAL, A. DABADE *

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: Histamine is a compound found in numerous foods and beverages which is an active allergen for human hypersensitive response. Naringenin is a colorless flavonoid used to reduce the histamine in wine. It has potential antibacterial, anti-inflammatory, antioxidant, anti-cancer, antifungal and inhibiting activity. The effect of 0.1mg^{-1} , 0.2mg^{-1} and 0.3mg^{-1} of naringenin on 30th day, 32nd day, 34th day, 36th day considered to reduce histamine in wine. Samples are treated with ultrasound at 50Hz and ultraviolet ray for 0min, 15mins and 30mins. Concentrations of naringenin in wine was analyzed for spectrophotometric assay. Sensory analysis were carried out by a 9-point hedonic scale from 55 untrained panelists. The significant histamine reduction observed with increasing concentration of naringenin. The wine containing 0.3mg^{-1} of naringenin on 36th day reduced the concentration of histamine by -71.02%. The wine containing 0.3mg^{-1} of naringenin treated with ultrasound at 50Hz and ultraviolet ray at 30mins were reduced to -79.52% and -41.61% respectively as compared to control without any rejection from the sensory panel.

KEYWORDS: Histamine, biogenic amines, naringenin, wine, ultrasound, ultraviolet rays.

*Corresponding Author: Mr. Ashish Dabade

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Biogenic amines form a group of undesirable natural components widespread in foods and beverages, e.g. scombroid fish, meat and meat products, cheeses, vegetable products, beer and wine. It can have antagonistic impact representing a health risk for sensitive people[1].

Histamine is one of the biogenic amine and is present in alcoholic drinks, which is found to be capable of triggering allergic and allergic like adverse responses. It is a compound that occurs naturally in your body. It's likewise found in numerous food and beverage, particularly fermented items. For instance, fermented cheddar, smoked meats, sauerkraut, wine, and beer have a tendency to be high in histamines. Typically, your body creates a protein called diamine oxidase (DAO) to separate histamine. In the event that your body doesn't deliver enough dynamic DAO, you may respond to histamine in food and drinks. For instance, potential indications include red and irritated skin, nasal clog, shortness of breath, stomach torment, headache/Migraines, Difficulty falling asleep, easily arousal, Hypertension, Vertigo or dizziness, Arrhythmia, or accelerated heart rate, Difficulty regulating body temperature, Anxiety, Nausea, vomiting, Abdominal cramps, Flushing, Nasal congestion, sneezing, difficulty breathing, Abnormal menstrual cycle, Hives, Fatigue, Tissue swelling[2]

Red wine has a tendency to have more elevated amounts of histamine than white wine. White wine

contains 10 mg of histamine per litre, while red wine contains 30 mg of histamine per litre [3] Naringenin is colorless flavonoid found in fruits like grapefruit, bergamot, sour orange, tart fruits, tomatoes, cocoa, Greek oregano, water mint and additionally in beans. It has been shown to have antioxidant, anti-inflammatory, antiviral and inhibitory activity [4]

Objective in current study was to develop a simple and rapid chemical method for determination of histamine in wine and use it as an asset in assessing the quality of wine and later decrease the quantity of histamine by treating the fermented wine.

2. MATERIALS AND METHODS

2.1.Fermentation of wine

Approximately 3.5kgs of fresh pulpy, tender red grapes were washed, destemmed and crushed followed by filtration process. The filtered juice was admixed with 2 grams of KMS solution. Assurance of 20°Brix was observed using refractometer. 2.5 grams of EC-1118 dried yeast was activated, added to the filtered juice and agitated thoroughly. pH was adjusted between 3.8 to 4 by tartaric acid. The above juice was transferred into four different fermenting jar, 500ml each. Jar 'A' contained only 500ml of the juice, while Jar 'B' contained 50mg of naringenin in the 500ml juice, Jar 'C' contained 100mg of naringenin in the 500ml juice and Jar 'D' contained the highest concentration i.e, 150mg of naringenin in the 500ml juice. Each of the fermenting jars was kept for 30 days in room temperature. After 30 days, 20 ml of the wine was drawn out from each of the jars followed by the filtration process for further preparation of sample.

2.2.Spectrophotometric modification for histamine detection

2.2.1. Decolorization of wine

For every 20ml wine sample, 500mg of activated Carbon was added, stirred and allowed to rest for 2mins followed by filtration giving a colorless wine sample [5].

2.2.2. Development of new assay

p-phenyldiazonium sulfonate was prepared with minor alterations. 1.5ml chilled 0.9% of sulfanilic acid made with the diluent 4% Hydrochloric acid with 1.5ml 5% Sodium Nitrite were mixed in 50ml standard flask and kept in ice bath for 10mins. Post ice bath, 6ml of 5% Sodium Nitrite was added and the volume was made up with chilled distilled water. The reagent is then stored in ice bath for 15 mins and is only stable for 12h. In a clean, dry test tube 5ml of 1.1% Sodium Carbonate was taken to which 2 ml of the chilled reagent is added and mixed slowly. It was then added to the tube containing 1ml of the decolorized wine sample. The whole setup was allowed to remain at rest for 5 mins. The absorbance of the color produced was measured at 496nm [6].

2.2.3.Preparation of standard

Histamine Dihydrochloride ($C_5H_{11}Cl_2N_3$) is used for the preparation of standard. The range of standard concentration was from 10 μ g to 70 μ g and was prepared by serial dilution. After the preparation, 1ml of each is taken and later carried out by the above new assay development method (2.2.2)

2.3.Treatment

2.3.1.Treatment with naringenin

As per the above decolorization technique stated in (2.2.1), 1 ml of the decolorized wine sample from jar 'A' 'B' 'C' and 'D' was taken in each test tubes respectively. In a clean test tube, 5 ml of 1.1% Sodium Carbonate was taken to which 2ml of prepared chilled reagent was added and mixed slowly (2.2.2). It was then added to the test tube containing 1 ml of the decolorized wine sample mentioned above. The absorbance of the color produced was measured at 496nm.

2.3.2.Ultrasonic treatment with naringenin at 50Hz

Wine purchased from local market was transferred into 4 different beakers, 100ml each. Beaker 'A' contained only the wine, 10mg of naringenin was added to beaker 'B' containing 100ml of the wine, 20mg of naringenin added to the beaker 'C' containing 100ml wine and 30mg of naringenin added to beaker 'D' containing 100ml wine. Each of them were treated with ultrasound for 15 and 30mins in Ultrasonicator. After the treatment, 20ml from each was taken for decolorization as explained above (2.2.1). The decolorized sample was further used for assay (2.2.2). Sample untreated with ultrasound was also decolorized with same method and its absorbance was noted to record the reduction difference of histamine from the treated sample.

2.3.3.Ultraviolet treatment with naringenin

Wine purchased from local market was transferred to 4 different beakers, 100ml each. Beaker 'A' contained only the wine, 10mg of naringenin was added to beaker 'B' containing 100ml of the wine, 20mg of naringenin added to the beaker 'C' containing wine and 30mg of naringenin added to beaker 'D' containing wine. Each of them were treated with ultraviolet ray by exposing the samples to UV lamp from top, under enclosed conditioned for 15 and 30mins. After the treatment, 20ml from each was drawn for decolorization as explained above (2.2.1). The decolorized sample was further used for assay (2.2.2). Sample untreated with ultraviolet ray was also decolorized with same method and its absorbance was noted to record the reduction difference of histamine from the treated sample.

2.4.Sensory analysis

Organoleptic based 9-point hedonic scale sensory analysis was carried out from 55 non-trained panels for acceptability of wine samples. Hedonic panel analyzed color, aroma, flavor, appeal, mouthfeel, and overall acceptability). 1=dislike extremely, 2=dislike very much, 3=dislike moderately, 4=dislike slightly, 5=neither like nor dislike, 6=like slightly, 7=like moderately, 8=like very much, 9=like extremely.

2.5.Color analysis

The samples were also analyzed for its colorimetric properties with assistance of research lab tools software 'Color Analysis'.

3. RESULTS AND DISCUSSION

Samples containing 10mg, 20mg and 30mg of naringenin drawn out for spectrophotometric assay on 30th day, 32nd day, 34th day and 36th day (table no.1) were observed to have significant difference with untreated control samples ($P>0.05$). Samples analyzed on 36th day was found to give maximum reduction (Fig.1).

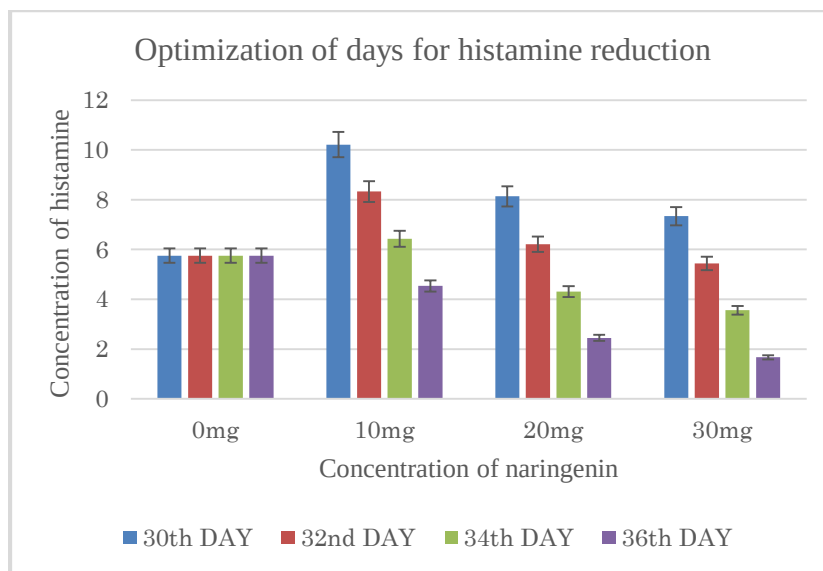


Figure 1: Optimization of days for histamine reduction

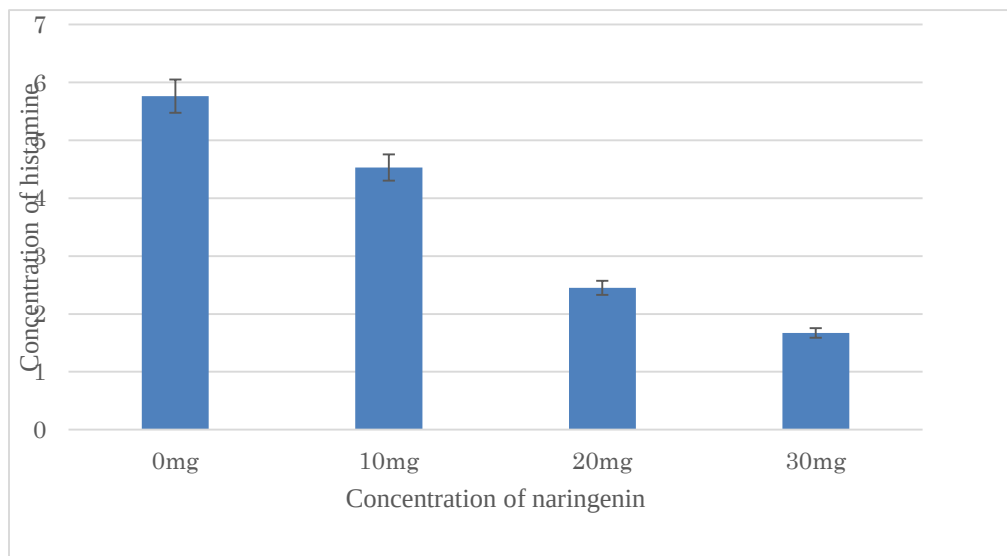


Figure 2: Optimization of concentration of naringenin for histamine reduction on 36th day

Samples containing 0mg, 10mg, 20mg and 30mg of naringenin was drawn out for spectrophotometric assay on 36th day, observed to have significant difference with untreated control samples ($P>0.05$). Samples analysed for 30mg of naringenin was found to give maximum reduction.

Hence, Sample containing 30mg of naringenin was observed to give maximum reduction on 36th day (Fig.2).

3.1.Ultrasonic treatment with naringenin at 50Hz

3.2.Optimization of time for histamine reduction

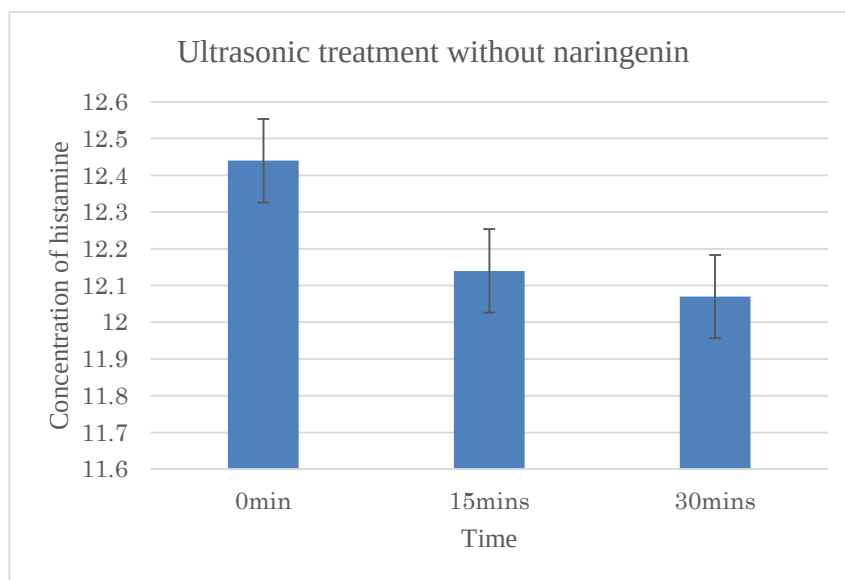


Figure 3: Optimization of time for histamine reduction by ultrasonic treatment at 50Hz

Samples treated with ultrasound at 50Hz were observed to have significant difference with untreated control samples ($P>0.05$). Samples treated with ultrasound at 50Hz for 30mins was found to give the maximum reduction (Fig.3).

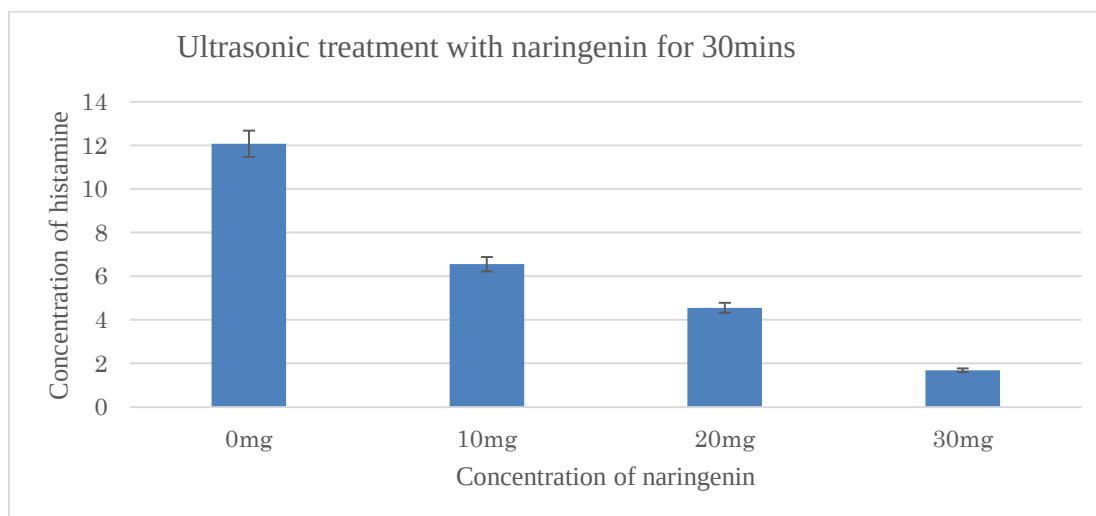


Figure 4: Optimization of concentration of naringenin for histamine reduction in 30mins by ultrasound at 50Hz

Samples treated with ultrasound at 50Hz were observed to have significant difference with

untreated control samples ($P>0.05$). Samples containing 30mg of naringenin treated with ultrasound at 50Hz was found to give the maximum reduction (Fig.4).

Hence, Sample containing 30mg of naringenin treated with ultrasound at 50Hz for 30mins were opted (Fig.4).

3.3.Ultraviolet treatment with naringenin

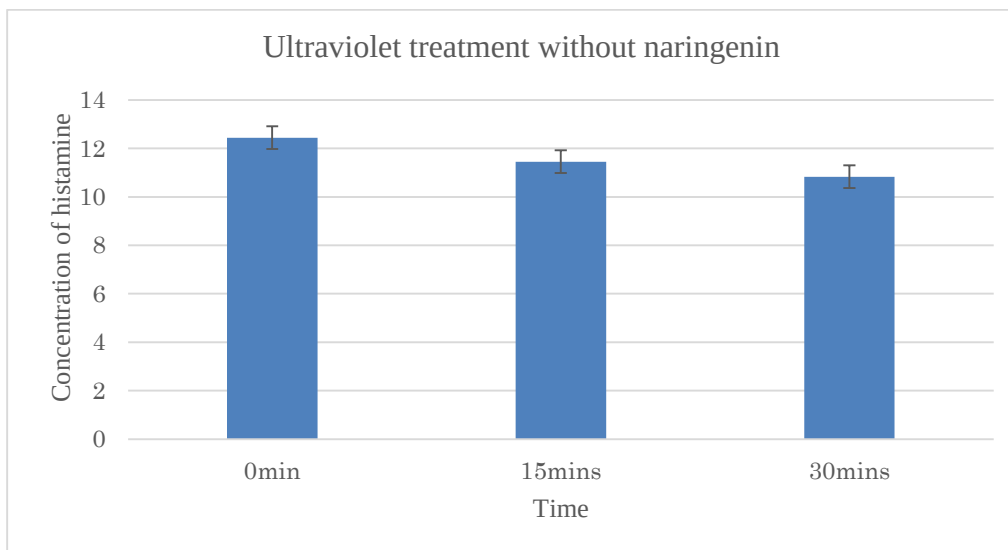


Figure 5: Optimization of time for histamine reduction by ultraviolet treatment

Samples treated with ultraviolet ray were observed to have significant difference with untreated control samples ($P>0.05$). Sample treated with ultraviolet rays for 30mins was found to give the maximum reduction (Fig.5).

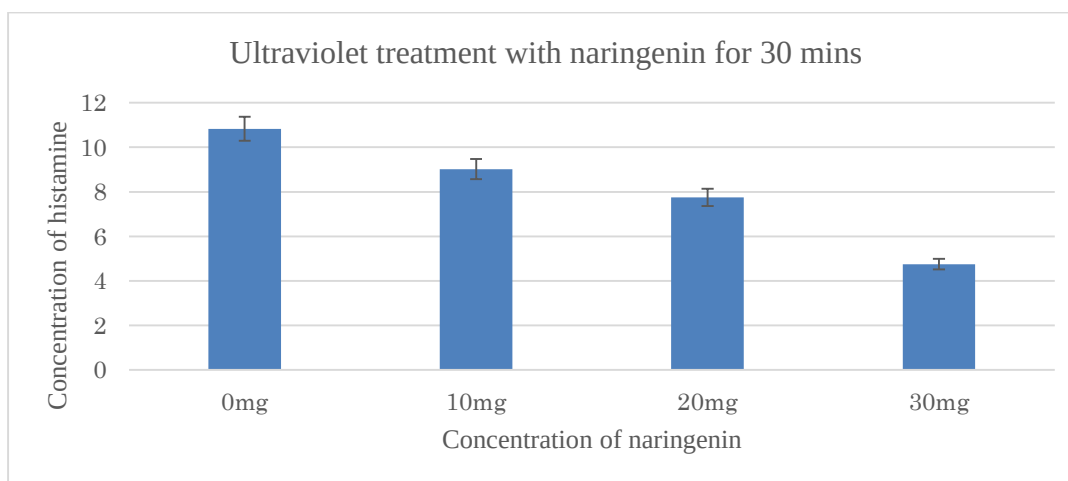


Figure 6: Optimization of concentration of naringenin for histamine reduction in 30mins by ultraviolet treatment

Samples treated with ultraviolet ray were observed to have significant difference with untreated

control samples ($P>0.05$). Samples containing 30mg of naringenin treated with ultraviolet ray was found to give the maximum reduction (Fig.6).

Hence, Sample containing 30mg of naringenin treated with ultraviolet ray for 30mins were opted (Fig.6).

3.4. Comparison between treatment by naringenin, ultrasonic treatment by naringenin and ultraviolet treatment by naringenin

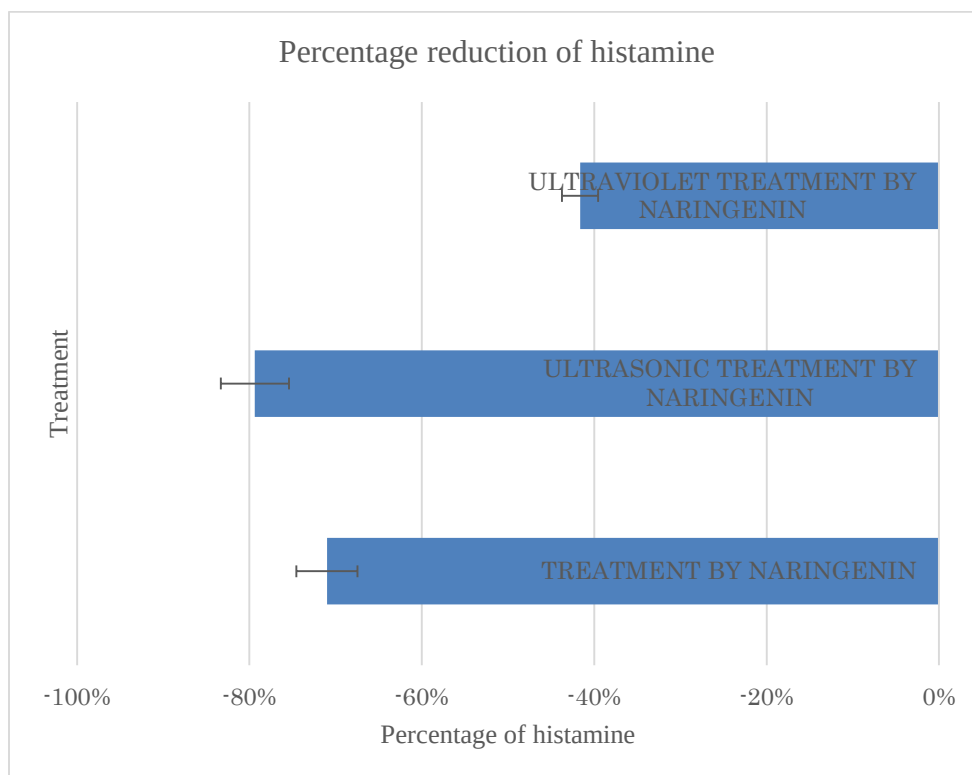


Figure 7: Comparison between treatment by naringenin, ultrasonic treatment by naringenin and ultraviolet treatment by naringenin

Samples with naringenin and samples with naringenin treated by ultrasound at 50Hz and ultraviolet ray were observed to have significant difference with untreated control samples ($P>0.05$). Samples with ultrasound at 50Hz was found to give the maximum percentage reduction (Fig. 7).

3.5. Sensory Analysis

Panelist did not reject any sample in sensory evaluation. The wine samples with naringenin analyzed on 36th day (Fig.8), also treated with ultrasound at 50Hz (Fig.9) and ultraviolet ray (Fig.10) were accepted by the sensory panel without any objection. Which shows that all the parameters in sample remained unaltered. 9-point hedonic scale sensory evaluation based on color, aroma, mouthfeel, flavor, appeal, overall acceptability by untrained panel highly accepted.

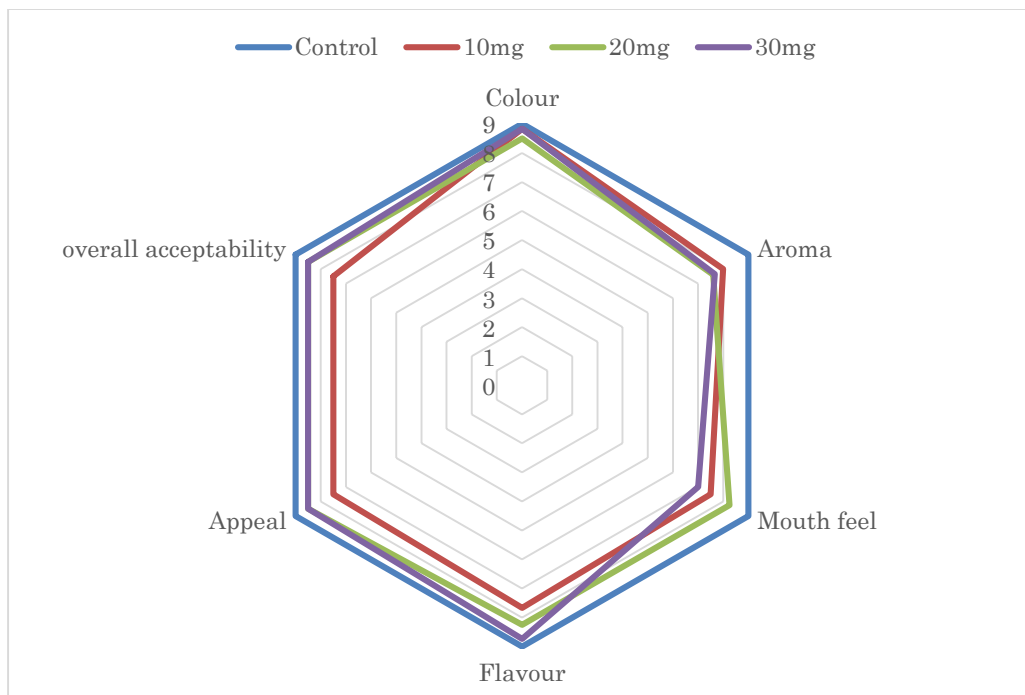


Figure 8: Radar graph for 9-point hedonic scale sensory evaluation based on color, aroma, mouthfeel, flavor, appeal, overall acceptability by the untrained panel for the sample with naringenin on 36th day

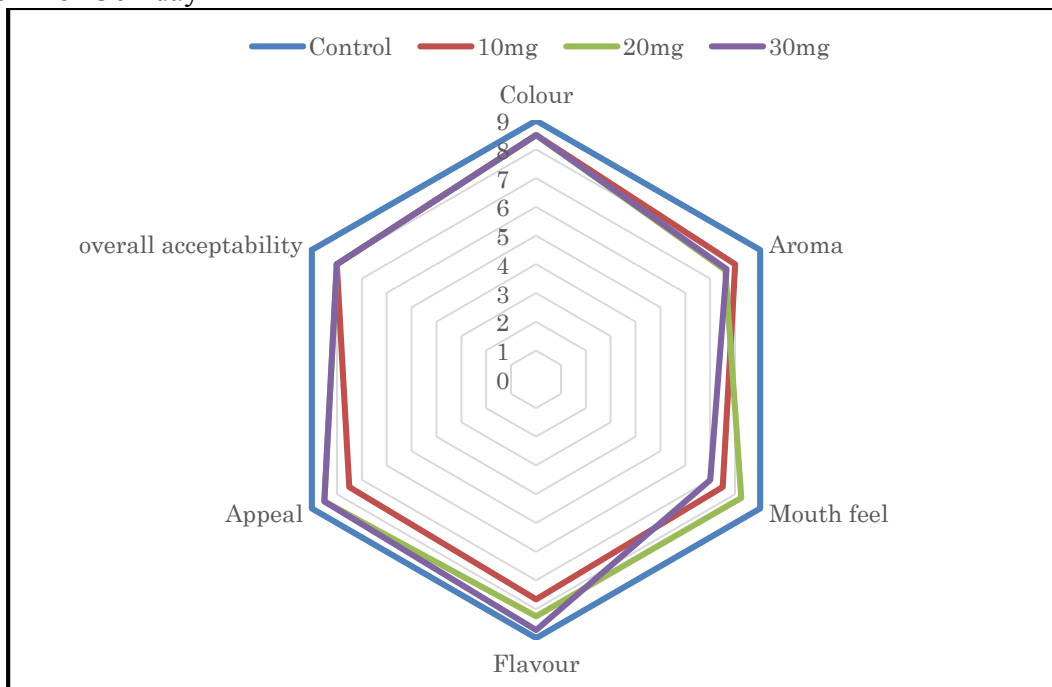


Figure 9: Radar graph for 9-point hedonic scale sensory evaluation based on color, aroma, mouthfeel, flavor, and overall acceptability by the untrained panel for the sample treated with ultrasound at 50Hz

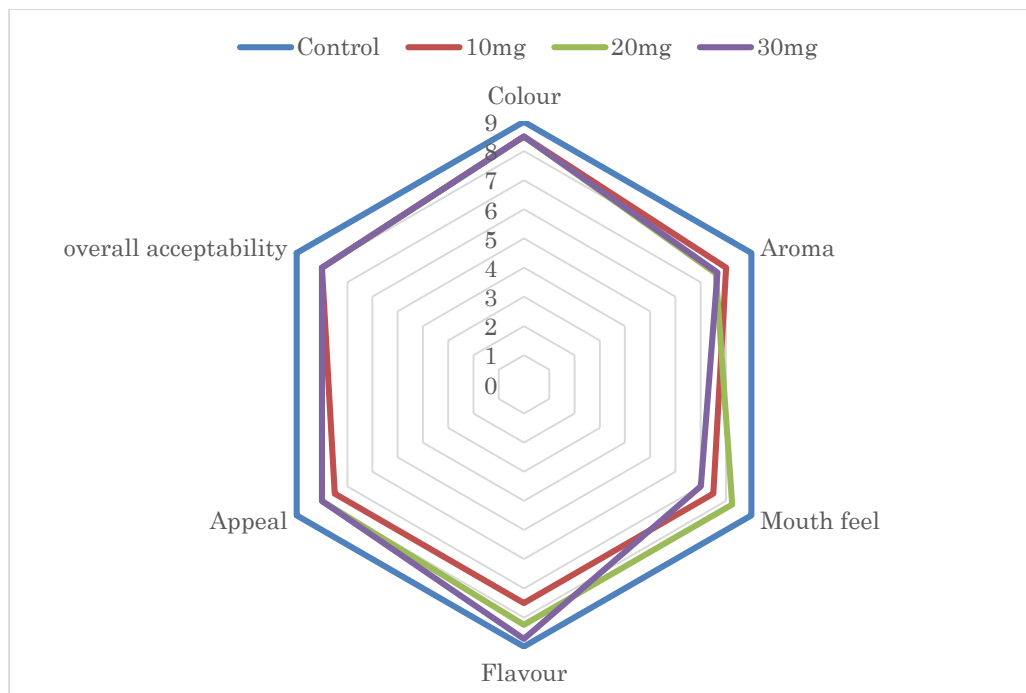


Figure 10: Radar graph for 9-point hedonic scale sensory evaluation based on color, aroma, mouthfeel, flavor, appeal, and overall acceptability by the untrained panel for the sample treated with ultraviolet ray

3.6.Color Analysis

The samples with naringenin on 36th day (Fig.11) and the samples with naringenin given ultrasound (Fig.12) and ultraviolet treatment (Fig.13) shows no distinctive color change.

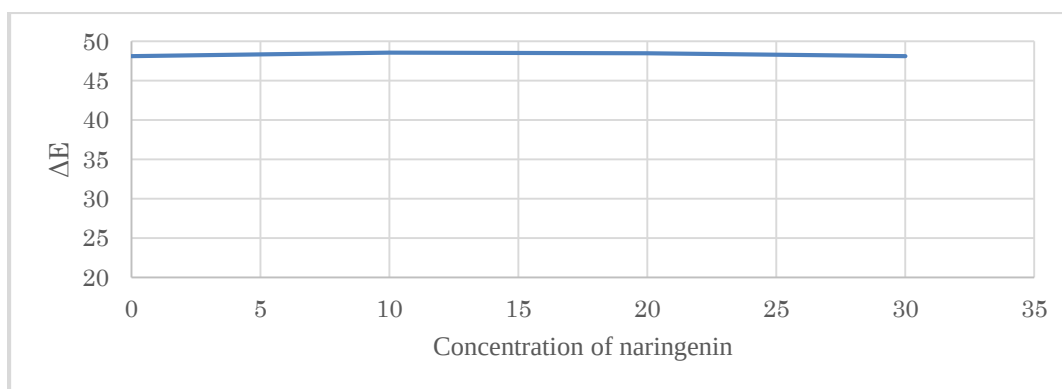


Figure 11: Color Analysis on wine with naringenin on 36th day

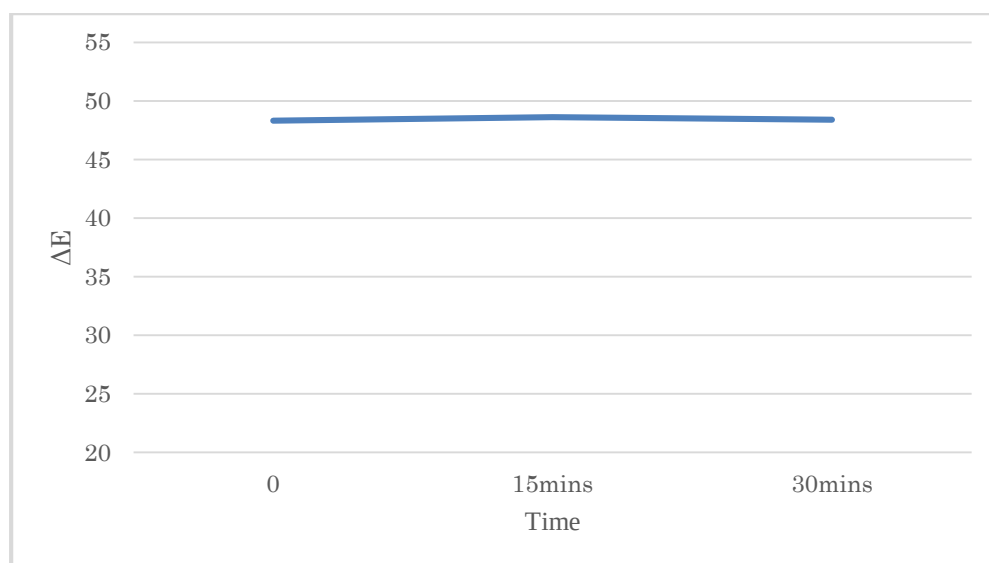


Figure 12: Color Analysis on wine with naringenin by ultrasonic treatment

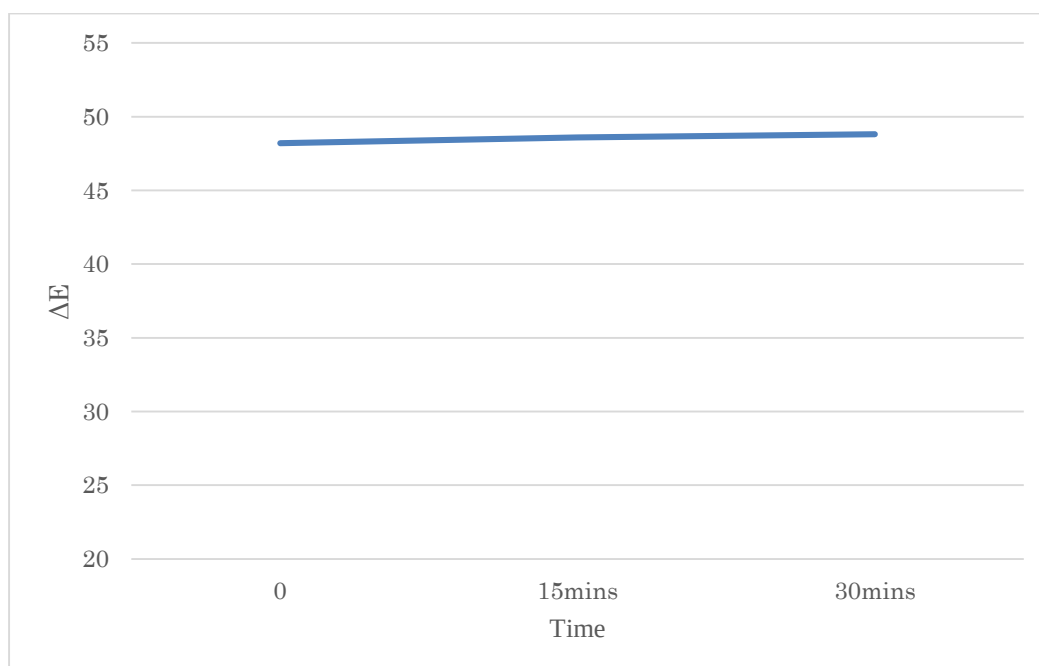


Figure 13: Color Analysis on wine with naringenin by ultraviolet treatment

Days	Concentration of histamine (μg)			
	0mg Naringenin	10mg Naringenin	20mg Naringenin	30mg Naringenin
30 th	5.75±0.00 ^a	10.21±0.001 ^d	8.13±0.01 ^d	7.33±0.01 ^d
32 nd	5.75±0.00 ^a	8.32±0.02 ^c	6.20±0.01 ^c	5.44±0.01 ^c
34 th	5.75±0.00 ^a	6.42±0.01 ^b	4.30±0.01 ^b	3.55±0.01 ^b
36 th	5.75±0.00 ^a	4.53±0.01 ^a	2.44±0.01 ^a	1.66±0.01 ^a

Table 1: Optimization of Days for Histamine Reduction (N=3)

4. CONCLUSION

The formulated method for reduction of histamine from wine was carried out by means of variation in treatments. Post sensory analysis, 30mg of naringenin was proven to give satisfactory results without affecting its sensory parameters. The study for reduction in histamine by naringenin was found to be positive by giving fair reduction in the concentration by -71.02%. The treatment with ultraviolet ray along with naringenin was also found to reduce the concentration of histamine by -41.61%. However, the treatment of ultrasound at 50Hz along with naringenin proved to give maximum percentage reduction in the concentration of histamine by -79.52%.

Wine being one of the fermented drinks, has a very complex pallet for sensory hence, it is susceptible to off flavors with higher chances of affecting the sensory. With all these factual considerations, the studies were designed and carried out to not affect any sensory parameters.

The study for the colorimetric analysis for the wine samples was also carried out and found to show no distinctive change.

5. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Functional Properties of De-Oiled Rice Bran Protein as a Potential Emulsifier-A Solution to Rice Bran Oil Industry

M. CHUGULE, S. KAMAT, S. SHITOLE, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: De-oiled rice bran is the major waste from rice bran oil industry. De-oiled rice bran contains 18.1% protein. Protein extraction was carried out 3 methods. Phenolic extraction method was found cost effective method with respect to protein yield. This protein was further used to study functional properties for food additives. Emulsion activity and stability were determined by UV-Visible spectroscopy at 500 nm. Determination of foaming capacity and foaming stability were carried out by high speed whipping foam generation method. Water holding and oil holding capacity of protein were determined by centrifugation method. Emulsion activity of de-oiled rice bran and soy lecithin was found 0.192 ± 0.002 and 0.180 ± 0.005 respectively. Emulsion stability of de-oiled rice bran was 0.154 ± 0.002 with stability time 10.6 minutes whereas soy lecithin stability was 0.178 ± 0.003 with stability time 10.1 minutes. Foaming capacity and stability were found 41% and 97% respectively. The de-oiled rice bran water holding capacity and oil holding capacity were 1.65 gm and 1.79 gm respectively whereas for soy lecithin water and oil holding capacity were 0.79gm and 0.42 gm respectively. The properties like emulsifying activity, emulsifying stability, foaming activity, foaming stability, water and oil holding capacity can be further use for various food additives purposes like emulsifier, foaming agent or water activity stabilizer or edible humectant.

KEYWORDS: Protein extraction, Water, oil holding capacity, Emulsion activity, stability, foaming capacity, and stability, food additives

***Corresponding Author: Mr. Ashish Dabade**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Rice (*Oryza sativa*) contains 80% carbohydrates, 7-8% proteins, 3% fat and 3% fiber and rice grain contains 5% bran which 12-18.5% is oil [1].

In recent years, about 610 million metric tons of rice are produced annually in Asian countries. This huge amount of production results in large amount of rice byproducts. The De-oiled bran, contain high amount of protein due to extraction of oil. It has not been utilized to its full potential [2].

Rice bran is a major by-product of rice milling industry with world production of about 50 to 60 million metric tons (MMT) per year. Rice bran generally used as cattle and poultry feed or simply discarded. 50% i.e. around 25-30 MMT of total bran use to produce rice bran oil leaving De-oiled

meal as the byproduct. About 10 MMT of rice bran produce in India annually which generate 5-6 MMT de-oiled rice bran annually[3]

A number of potential food uses of full fat and defatted rice bran for the preparation of bread, muffins, pancakes and cakes have also been reported. De-oiled rice bran protein has been reported to be of high quality which has unique nutritional value and nutraceutical properties. The protein content of De-oiled rice bran is high in lysine content than rice endosperm protein or any other cereal bran proteins[4].

2. MATERIALS AND METHODS

2.1.Chemicals and Reagents

De-oiled rice bran was obtained from M/s A. S. Nutra Tech Pvt. Ltd. (Unit- 2), Nadi More, Village-Ghodari, Mahasamund, Chhattisgarh state. Rice bran stored at 4°C until used. Soy lecithin and sunflower oil were purchased from a local vendors. Phenol, Tris-HCl, EDTA, sucrose, KCl, 2%β-mercaptoethanol, Phenyl-methyl-sulfonyl Fluoride, Ammonium Acetate, Methanol, Sodium deodyl sulphate, Trichloroacetic acid, Sodium Carbonate, Sodium potassium tartrate, Copper sulphate were ordered from SRL laboratories. Folin-ceocaltu reagent was ordered from Sigma Aldrich, Distilled water, Sunflower oil (Brand-saffolla), Liquid Nitrogen (Trpti Gases).

2.2.Methods

2.2.1.Proximate analysis

Proximate analysis of DE-OILED rice bran in terms of moisture content, crude protein, crude fat, ash and crude fibre were estimated [5].

Protein yield comparison for different Extraction: Method A- The protein was extracted by phenolic method described in[6]. Method B- The protein was extracted by TCA [7]. Method C- The protein was extracted by phenolic extraction method[8].

2.2.2 Protein content analysis

De-oiled rice bran protein yield was analyzed by standard curved developed by spectrophotometer (UV- VIZ Shimadzu, Japan) based on Folin- Lowry method [5].

2.2.3 Emulsion Preparation

Oil-in-water type emulsions were prepared with some modifications[9]. Oil and water type emulsions were prepared with soy lecithin and De-oiled rice bran protein. 0.5gm 5ml sunflower oil (Suffola Brand) and 5ml distilled water mixed with various concentration 1%, 2%, 3%, 4%, 5% de-oiled rice bran protein concentration and compared with soy lecithin concentrations. Emulsification using homogenizer was carried at 8,000 rpm for 1 minute.

2.2.4 Emulsifying activity

Emulsifying activity were determined using by with some modifications[10]. The 1%, 2%, 3%, 4% and 5% emulsions were diluted with 5ml of 0.1% (w/v) sodium dodecyl sulfate (SDS) solution.

The absorbance of the emulsion was measured at 500 nm by spectrophotometer (UV- VIZ Shimadzu, Japan)

2.2.5 Emulsion stability

Emulsion stability index were determined using the method described in [11]. 50ml of emulsion was diluted with 5ml of 0.1% (w/v) SDS solution and the absorbance at 500 nm was measured by spectrophotometer. Emulsion stability index was calculated according to the equation Emulsion stability (min) = $A_0 \div \Delta A$

Where, A_0 is the absorbance at 0 min; ΔA is the change in absorbance during the time interval and t is time.

2.2.6 Foaming Capacity

Foaming capacity and foaming stability were determined [12]. Different concentration that is 1%, 2%, 3%, 4%, 5% of de-oiled rice bran proteins were prepared in distilled water. Whipping method was carried out with blender (Bajaj FX10, India). The blended sample immediately transferred to measuring cylinder.

$$\text{Foaming capacity \%} = \frac{\text{Volume after blending-volume prior to blending}}{\text{Volume prior to blending}} \times 100$$

2.2.7 Foaming stability

Foaming capacity and foaming stability were determined according to the method modified from [12]. The samples allowed to stand for 30 min at room temperature to estimate the foaming stability given by the formula

$$\text{Foaming Stability \%} = \frac{\text{Residual foam volume}}{\text{Total foam volume}} \times 100$$

2.2.8 Water holding capacity

The water holding capacity was determined centrifugation method as mentioned in [12] The water holding capacity = Final weight after centrifugation – Initial weight before centrifugation.

2.2.9 Oil holding capacity

The oil holding capacity was also determined by centrifugation method as mentioned in [12]

3. RESULTS AND DISCUSSION

3.1. Protein Yield

De-oiled rice bran proximate analysis shows 18.1% total protein (Table. 1).

3.2. Protein Extraction Method

Among the 3 methods applied for protein extraction; Method C found efficient in which 16% protein yield can be extracted (Table.2). Method C can give protein extraction yield up to 88.39%

Method A and Method B can extract protein up to 16.57% and 55.24% respectively. Method C can use at industrial level to extract the protein from de-oiled rice bran.

3.3.Emulsifying activity

Rice bran protein showing good emulsification properties similarly like soy lecithin (Figure.1). The rice bran protein and lecithin emulsifying activity comparison shows that rice bran protein can be used as lecithin replacer. The emulsifying activity of rice bran protein was 0.192 ± 0.002 whereas the emulsification activity of soy lecithin was 0.180 ± 0.005 at 5% emulsifier concentration (Figure.1).

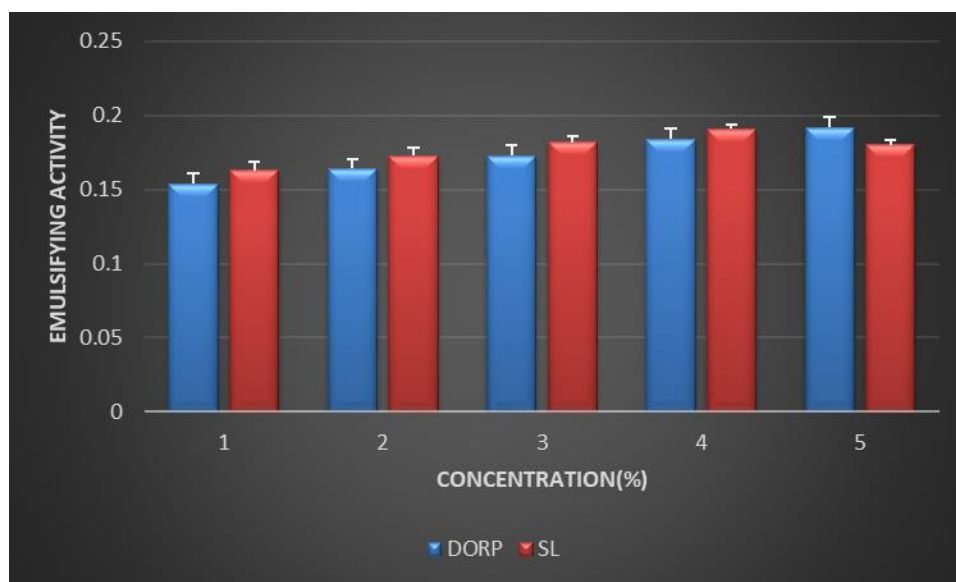


Figure 1: Emulsifying Activity of de-oiled rice bran protein and soy lecithin.

DORP- De-oiled rice bran protein, SL- Soy Lecithin

3.4.Emulsifying Stability

Emulsifying stability of rice bran protein shows no significant difference with soy lecithin ($P > 0.05$). The 5% concentrated rice bran protein shows similar stability as compared to soy lecithin (Figure 2). The de oiled rice bran protein also shows similar emulsification property as compared to soy lecithin.

3.5.Foaming capacity and stability

Foaming capacity and stability values of de oiled rice bran protein represent properties of good foaming agent. 5% de oil rice bran protein was showing foaming capacity and stability 41% and 97% respectively. The rice bran protein foaming capacity and stability can be use in various food products as foaming agent.

Water and Oil holding capacity- The significant difference ($P < 0.05$) found in water and oil holding capacity of rice bran protein and soy lecithin. Water and oil holding capacity of de-oiled rice bran protein was 3.3gm/gm protein and 3.58gm/gm protein. The 108% more water holding capacity of de oiled rice bran protein than soy lecithin was found. This property of protein can be use as

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softening agent for various products like bread.

Ultimately de-oiled rice bran protein has promising properties like emulsifier, foaming and softening agent. De-oil rice bran is considered as food cum oil industry waste. This waste has very negligible value than soybean seeds. The low cost raw material ultimately serves low cost emulsifier. Currently most of the food industries prefers soy lecithin as a natural emulsifier under INS number E 322. The de oiled rice bran protein shows non-significant effect with soy lecithin. The industries can replace the soy lecithin with de oiled rice bran as emulsifier. (Figure 3)



Figure 2: Emulsifying stability of de oiled rice bran protein and soy lecithin.

DORP- De-oiled rice bran protein, SL- Soy Lecithin

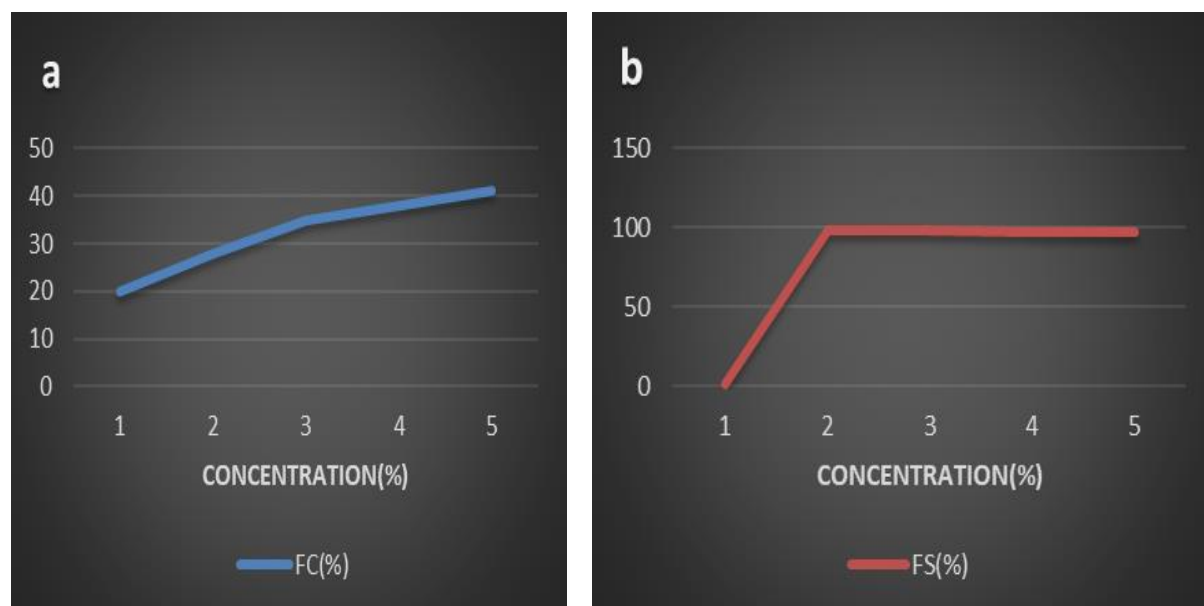


Figure 3: (a) Foaming capacity (FC) (b) Foaming stability (FS)

Composition	Content %
Moisture	7.11
Ash	12.7
Protein	18.1
Fat	5.8
Crude Fibre	13.9
Carbohydrate	56.27

Table 1: Proximate Analysis of De-Oiled Rice Bran.

Method	Protein Yield in %
Method-1	3
Method-2	10
Method-3	16

Table 2. Protein Yield Comparison for Different Extraction

Composition	de-oiled rice bran protein gm/gm protein	Soy lecithin gm /gm protein
Water Holding Capacity	3.3	1.58
Oil Holding Capacity	3.58	0.84

Table 3: Water Holding and Oil Holding Capacity

4. DISCUSSION

The rice bran extracted protein can be used as a replacement for the various emulsifiers and foaming Agents.

The utilisation of waste from rice bran oil industry can also provide the new solution to reduce the waste production from rice bran oil industry.

5. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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A Study on Development of Functional Beverages from Under - Utilized Fruits

V. CHAWLA, N.S. BHALERO, SHANOoba.P.M*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: Mixed fruit juices are widely accepted as compared to pure or concentrated fruit juices due to their natural flavors, availability and health benefits. Moreover, pure or concentrated juices are unacceptable to various consumers because of their astringent flavor and taste in many cases. Two functional beverages were developed by blending Jackfruit- Papaya fruit juice and Pomegranate-Jamun fruit juice. The blends were optimized based on pH, TSS, Total titratable acidity, Vitamin C content, antioxidant properties, total phenolic contents and flavonoid content and sensory analysis against pure juice controls. Out of the different combinations such as 70:10, 60:20, 50:30, 40:40 and 30:50, in Pomegranate - Jamun blend combination B (60:20), showed the highest antioxidant activity, flavonoids content, total phenolic content. The overall acceptability of this combination was also significantly higher compared to other combinations. For Jackfruit - Papaya blend, combination A (70:10) showed the highest antioxidant activity, flavonoids content, total phenolic content and overall acceptability compare to other combinations. The optimized Pomegranate - Jamun blend was further analyzed to evaluate the effect of its pH on color stability and anthocyanin content. Also, the effect of various stabilizers on Pomegranate - Jamun blend was studied.

KEYWORDS: functional beverages, antioxidants, titerable acidity, turbidity

***Corresponding Author:** Dr. Shanooba P. M.

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: shanooba.p.m@dypatil.edu

1. INTRODUCTION

Fruit juice blends are highly refreshing, thirst quenching, appetizing, easily digestible and nutritious, as they are rich sources of vitamins, fiber and/or mineral salts for human consumption (Sethi, Sethi, Deka, & Meena, 2005). Fruit juices, which are naturally rich in bioactive compounds with health promoting and disease-reducing properties, are important contributors to human nutrition. These natural health promoting bioactive components provide consumers with a large variety of health benefits, such as maintaining normal blood pressure and protecting the skin, bones, cardiovascular, and nervous system (Ephrem, Najjar, Charcosset, & Greige-Gerges, 2018). In turn, they are far superior to synthetic drinks available in the market. Blending of fruit juices is adjudged one of the best methods to improve the nutritional quality of the juice. The vitamin and mineral contents of the juice can be improved, depending on the kind and quality of fruits used (De Carvalho, Maia, De Figueiredo, De Brito, & Rodrigues, 2007). Mixed fruit beverages are obtained by blending two or more, appropriately selected fruit juices combined in varying proportions.. Moreover, blending is the best way for the judicious use of under-utilized yet high potential,

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indigenous fruits, which are generally discarded or overlooked owing to their very low total soluble solids (TSS), excessively strong flavor, high acidity, astringency and bitterness. On the other hand, blending may result in a new natural drink which is also capable of serving as an appetizer. Among the new products, blended fruit juices (BFJs) stand out to enhance the sensorial and nutritional characteristics of these products. Mixing fruit juices provides increased concentrations of selected bioactive compounds, adds new nutrients, or improves flavor and appearance. Besides, it has been reported that absorption of bioactive compounds in fruit juices exceeds that after consumption of intact fruits. Therefore, the bioavailability of these substances could be also enhanced through BFJs. (Jayachandran, Chakraborty, & Rao, 2015)

Pomegranate

Pomegranate fruit (*Punica granatum*) has been used as a food and traditional medicine (Still, 2006). The polyphenols in pomegranate have antioxidant activity (Schubert et al., 1999; Noda et al., 2002). In fact, pomegranate juice contains more polyphenols than red wine, blueberry, cranberry, green tea, or orange juice (Aviram, 2002), and its antioxidant activity can be as much as three times higher than red wine or green tea (Gil et al., 2000). In human clinical research, pomegranate ellagitannin-enriched polyphenol extract significantly reduced the production of thiobarbituric acid reactive substances (TBARS) in plasma (Heber et al., 2007). Furthermore, human (Rosenblat et al., 2006) and animal (Kaplan et al., 2001) researches suggest that pomegranate juice may slow the progression of atherosclerosis, in association with reduced lipid peroxidation of low-density lipoproteins (LDL). (Bruno, 2016)

Jamun

Jamun (*Syzygium cumini*) commonly known as Indian blackberry, is an underutilized fruit from the Indian subcontinent and it belongs to the Myrtaceae family. The jamun fruits are available abundantly during the summer season for a short period. The fruits are deep purple or bluish in colour with pinkish pulp and are widely consumed as a fruit and also used for the treatment of various diseases as an astringent, antiscorbutic, diuretic, antidiabetic, and in chronic diarrhea and enlargement of the spleen (S. Achrekar, G.S. Kaklij, M.S. Pote, S.M. Kelkar, 1991). Its fruit is a rich source of anthocyanins whose content is equivalent to that of blueberries and black currants and higher than that of blackberries, all widely acclaimed anthocyanin-rich edible fruits. The fruits are edible and are reported to contain vitamin C, gallic acid, tannins and anthocyanins including delphinidin, cyanidin, petunidin, malvidin-glucoside and other components, which are responsible for the deep purple colour (A. Banerjee, N. Dasgupta, B. De, 2005) These beneficial effects are mostly due to the presence of bioactive compounds, such as pigments and phenolic compounds (A. Chaturvedi, G. Bhawani, P.K. Agarwal, S. Goel, A. Singh, R.K. Goel, 2009)

Jackfruit

Jackfruit (*Artocarpus heterophyllus*) is a large-fruit crop (up to 10 kg) and widely distributed throughout the tropics and subtropics. Jackfruit is very popular with the people who live in the southern part of China. The pulp of the ripe fruit is commonly eaten fresh and also processed into various products such as canned products, dried fruit, juice, and baked goods. About 60% of the

whole fruit is non-edible, consisting of prickly rind, inner non-edible perianth and a central core, which comprise non-utilized waste (Subburamu, Singaravelu, Nazar, & Irulappan, 1992). Jackfruit peel contains some 8.94-15.14% dry weight of pectin (Begum, Aziz, Uddin, & Yusof, 2014). Accordingly, jackfruit peel as a good source of pectin, and the constructive utilization of jackfruit by-products, will be of great importance in the jackfruit processing industries. (Xu et al., 2018)

Papaya

Papaya is the plant of *Carica papaya* species from genus *Carica* of the family Caricaceae. The origin of papaya is in tropics of the Americas, from southern Mexico and neighboring Central America. Many potential bioactive components of papaya (vitamin A, ascorbic acid, a-tocopherol, B vitamins, flavonoids, and carotenes such as b-cryptoxanthin, b-carotene, and lycopene) and strawberry (ascorbic acid, ellagic acid, folic acid, and flavonoids such as anthocyanin, catechin, quercetin, and kaempferol) have significant synergistic potential (Basu, Nguyen, Betts, & Lyons, 2014; Krishna, Paridhavi, & Patel, 2008).

2. MATERIALS AND METHODS

Materials

Jackfruit was purchased from Chembur, Mumbai. Papaya and pomegranate was purchased from local food market in Belapur CBD. Jamun pulp was purchased from Suyog Food Products, Pune.

Methods

Extraction of Juices

About 300 g of pomegranate was weighed and 100 ml water was added and blended in mixer and filtered through muslin cloth. 300 g jamun pulp was weighed and 300 ml water was added and blended in mixer and filtered through muslin cloth. 300 g of jackfruit was weighed and 400 ml water was added and blended in mixer and was filtered through muslin cloth and for papaya juice 300 g was weighed and 300 ml water was added and blended in mixer and filtered through muslin cloth. The combinations were pasteurized at 90° C for 30 second. The combinations for pomegranate-jamun and jackfruit-papaya are as followed:

Sr. No.	Sample	Pomegranate (ml)	Jamun (ml)
1.	A	70	10
2.	B	60	20
3.	C	50	30
4.	D	40	40
5.	E	30	50
6.	F	80	-
7.	G	-	80

Table 1: Combination of pomegranate-jamun at different concentrations

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Total Soluble Solids (TSS)

TSS was determined by using hand refractometer (°Brix) (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

pH - pH was determined by using pH meter (Manik Chandra Roy, 2016)

Sr. No.	Sample	Jackfruit (ml)	Papaya (ml)
1.	A	70	10
2.	B	60	20
3.	C	50	30
4.	D	40	40
5.	E	30	50
6.	F	80	-
7.	G	-	80

Table 2: Combination of jackfruit and papaya at different concentrations

DPPH Radical Scavenging Activity Assay

Juice sample were diluted with distilled water 100:10000 μ l (0.1 ml: 10 ml). 0.5 ml of juice sample was added to 3 ml of 0.1 mM DPPH solution. The mixed solution was kept for 30 minute at ambient temperature in dark and the absorbance of solution was measured at 517 nm (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

Titrateable Acidity (TTA)

About 2 grams of juice was diluted in 100 ml distilled water. The sample was titrated against 0.1N NaOH and 2 to 3 drops of phenolphthalein indicator was added. The end point changes from colorless to pink (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

Total Phenolic Content (TPC)

About 0.5 ml sample was taken and 2.5 ml of 10% FC reagent was added. The mixed solution was kept at room temperature for 8 minute. In the solution 2 ml of 7.5% Na_2CO_3 was added and kept in dark at room temperature for 1hour. Absorbance was measured at 765 nm (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

Ascorbic Acid Content (AA)

About 10 ml juice was taken and 15 ml oxalic acid was added. The solution was titrated against

standard 2, 6-dichlorophenol indophenol dye (DCPIP). The endpoint changes from red to maroon (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

Flavonoids Content

The juice was diluted 1:100 with distilled water. 0.5 ml sample was taken and the volume was made up to 2 ml by using methanol (95%). 0.1 ml aluminium chloride (10%) was added to sample solution. 0.1ml of 1M potassium acetate was added and then 2.8 ml of distilled water was added and incubated at room temperature for 30 min. Absorbance was measured at 415 nm.

Sensory Evaluation

Sensory evaluation was carried out for pomegranate-jamun and jackfruit and papaya blends. A 15-member sensor panel was randomly chosen among students. All samples were randomly labeled alphabetically and the panelist evaluated each sample for taste, color, aroma and overall impression on a 9-point hedonic scale from one for dislike extremely to nine for like extremely (Oluwaseun P. Bamidele, Mofoluwaso B. Fasogbon, 2016)

Extraction of Pectin

Pomegranate peel was kept for drying at 60°C till the weight of dried peel was constant. Pectin extraction was performed using 0.1 N HCl solvent at 90°C at extraction time 120 min and pH 2.0. The solution pH was adjusted with HCl and NaOH. 5 g pomegranate powder was added in 150 ml 0.1 N HCl and heated in water bath for 2 hours. The heated extractant was filtered through muslin cloth and pressed to recover the extract. The pectin was precipitated by ethanol (95-98%) and kept at room temperature overnight. The precipitated pectin was filtered through Whatman No.1 and washed with 75% ethanol, 85% ethanol and absolute ethanol to remove the soluble impurities. Then, pectin was dried at 60°C for 24hr in an oven (Manik Chandra Roy¹ | Majbaul Alam¹ | Abu Saeid¹ | Bijoy Chandra Das¹ | Md. Biplob Mia¹ | Md. Atikur Rahman¹ | Jong Bang Eun² | Maruf Ahmed^{1, 2}, 2016)

Anthocyanin Content

The pH was adjusted to 2.5, 4.0, 6.0 and 8.0 using citric solution and NaOH. Then, the juice blend was heated to 90°C for 30 second. After cooling, the blend was diluted with pH buffer 1.0 (potassium chloride) and pH buffer 4.5 (sodium acetate). 1 ml blend was added with 5 ml of pH buffer 1.0 and 4.5. At pH buffer 1.0 the color of juice changes to pink and at pH 4.5 buffer the juice changes to colorless. Absorbance was measured at 520 nm and 700 nm respectively (Suthida Akkarachaneeyakorn & Sirikhwan Tinrat, 2014)

Effect of pH on color stability

The pH of pomegranate-jamun blend was adjusted to 2.5, 4.0, 6.0, and 8.0 using citric solution and NaOH. Then, the juice blend was heated to 90°C for 30 second. After cooling, the color of juice was measured (Suthida Akkarachaneeyakorn & Sirikhwan Tinrat, 2014)

Effect of Stabilizers

In pomegranate and jamun combination, commercial pectin, CMC (Carboxymethyl Cellulose) and extracted pectin was added at different concentrations i.e. 0.1%, 0.3% and 0.5%. Then, the combination was heated at 90°C for 30 second and cooled down to room temperature. The juice was stored at 4°C for 1 week and on days 0, 3, 5 and 7, the viscosity and turbidity was evaluated. The viscosity was measured by Brookfield viscometer and turbidity was measured by turbidity meter (Suthida Akkarachaneeyakorn & Sirikhwan Tinrat, 2014)

3. RESULTS AND DISCUSSION

Pomegranate-jamun combination

The combination for pomegranate-jamun was made with different concentrations i.e. 70:10, 60:20, 50:30, 40:40, 30:50 and 80 ml of pomegranate and jamun pure juice and was further analysed for different parameters.

TSS (°Brix), pH and Titratable acidity

For pomegranate and jamun combination the TSS, pH and TTA are as followed:

Sr. No.	Sample	Combination (Pomegranate:Jamun) (ml)	TSS (°Brix)	pH	Titrateable acidity (TTA)
1.	A	70:10	9.8	3.79	0.096 ± 0.21
2.	B	60:20	9.3	3.73	0.096 ± 0.21
3.	C	50:30	8.9	3.70	0.064 ± 0.07
4.	D	40:40	8.7	3.65	0.032 ± 0.14
5.	E	30:50	7.4	3.60	0.064 ± 0.14
6.	F	80	7.7	3.85	0.064 ± 0.14
7.	G	80	7.2	3.52	0.064 ± 0.14

Table 3: TSS, pH and TTA for pomegranate-jamun combination

The total soluble solid (TSS) of the juice blend was between 7.7 for pomegranate juice and 7.2

for jamun juice. The TSS of combination A and B increased which showed 9.8 and 9.3 as the concentration of pomegranate juice increased and in combination C and D the TSS decreased which showed 8.9 and 8.7 as the concentration of jamun juice increased. The pH ranged between 3.85 and 3.52 for pomegranate and jamun juice respectively. There was minute difference in pH on addition of jamun juice in pomegranate juice even blending ratios was different. The titratable acidity (TTA) of the juice blend was between 0.064 for pomegranate juice and 0.064 for jamun juice respectively. Combination A and B showed highest TTA than compared to other 3 samples. As there is high concentration of pomegranate juice in the blend it showed highest value of TTA. As the concentration of jamun juice increases the TTA of juice combinations decreased in remaining 3 combinations. However, incorporation of jamun juice in the blends increased TTA of the juice compared to pure pomegranate juice.

DPPH radical scavenging activity determination, Total phenolic content (TPC), Flavonoids content, Ascorbic acid content (AA)

For pomegranate and jamun combination the DPPH radical scavenging activity determination, total phenolic content, flavonoids content and ascorbic acid content are as follows:

Sr. No.	Sample	Combination (Pomegranate:Jamun) (ml)	DPPH ($\mu\text{g/ml}$)	TPC ($\mu\text{g/ml}$)	Flavonoids content (mg/100g)	Ascorbic acid content (mg/100ml)
1.	A	70:10	18.4549 ± 0.11	63.4649 ± 0.02	7.1875 $\pm$ 0.01	15.8076 $\pm$ 0.01
2.	B	60:20	17.089 ± 0.07	62.9825 ± 0.0	4.84375 $\pm$ 0.00	10.4811 $\pm$ 0.02
3.	C	50:30	16.471 ± 0.04	61.7654 ± 0.02	3.90625 ± 0.00	18.21306 ± 0.26
4.	D	40:40	16.6873 ± 0.00	63.9035 ± 0.0	9.21875 $\pm$ 0.00	32.64605 $\pm$ 0.32
5.	E	30:50	17.0519 ± 0.02	63.9035 ± 0.0	7.8125 $\pm$ 0.01	13.23024 $\pm$ 0.10
6.	F	80	14.2707 ± 0.03	64.4079 ± 0.03	6.25 $\pm$ 0.004	10.82474 $\pm$ 0.16
7.	G	80	15.6057 ± 0.00	64.4079 ± 0.03	4.84375 $\pm$ 0.00	10.82474 $\pm$ 0.14

Table 4: DPPH activity, TPC, Flavonoid content and AA of pomegranate-jamun combination

The DPPH radical scavenging activity for pomegranate juice was 14.2707 and 15.64057 for jamun juice. The combination a showed highest antioxidant activity i.e. 18.4549 compared to other 4 combination sample. In combination A 70 ml was pomegranate juice and 10 ml was jamun juice

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where it showed highest antioxidant activity. Because both the pure juice has antioxidant activity so, combination A showed highest antioxidant activity. The total phenolic content for pomegranate juice was 64.4079 and 64.4079 for jamun juice. Combination D and E showed highest TPC content compared to other 3 combinations. But pure juice of pomegranate and jamun showed higher TPC. The lowest TPC was recorded in combination C. The TPC of the juice blends were lower than pure pomegranate and jamun juice. The reduction in TPC value for the blend samples may be due to interaction between the phenolic compounds. The flavonoids content for pomegranate juice was 6.25 and 4.84375. The combination D showed highest flavonoids content followed by combination E and combination A. The lowest flavonoids content was found out in combination C. The combination D has equal concentration of pomegranate and jamun juice so it showed highest flavonoids content. In combination E the concentration of jamun was high as compared to pomegranate juice. But in combination A the concentration of pomegranate juice was high as compared to jamun juice. Both the juice has flavonoids present in them so, the flavonoid content was high in combination D followed by combination E and A. The ascorbic acid content (AA) for pomegranate juice was 10.82474 and 10.82474 for jamun juice. The combination D showed highest ascorbic acid i.e. 32.64605 which has equal concentration of pomegranate juice and jamun juice. Both the pure juices has ascorbic acid content present in them. The combination B showed low ascorbic acid content.

Jackfruit and papaya combination

The combination for pomegranate-jamun was made with different concentrations i.e. 70:10, 60:20, 50:30, 40:40, 30:50 and 80 ml of pomegranate and jamun pure juice and was further analyzed for different parameters.

TSS, pH and Titratable acidity (TTA)

Jackfruit and papaya combination the TSS, pH and titratable acidity (TTA) are as followed:

Sr. No.	Sample	Combination (jackfruit:papaya) (ml)	TSS (°Brix)	pH	Titrateable acidity
1.	A	70:10	7.2	4.95	0.096 ± 0.21
2.	B	60:20	6.8	4.44	0.08 ± 0.21
3.	C	50:30	6.6	4.19	0.08 ± 0.21
4.	D	40:40	5.6	4.17	0.064 ± 0.14
5.	E	30:50	5.0	4.15	0.128 ± 0.22
6.	F	80	8.0	4.16	0.112 ± 0.01
7.	G	80	3.5	5.03	0.08 ± 0.01

Table5: TSS, pH and TTA of jackfruit and papaya combination

The total soluble solid (TSS) of the juice blend was between 8.0 for jackfruit juice and 3.5 for

papaya juice. The TSS of combination A was highest as compared to other 4 combinations. As there was more concentration of jackfruit juice as compared to papaya juice so, the TSS of combination A increased. The pH ranged between 4.16 and 5.03 for jackfruit and papaya juice respectively. The similarity in the pH value may be attributed to equal amount of acid content of the juice combinations. There was minute difference in pH on addition of papaya juice in jackfruit juice even blending ratios was different. The titratable acidity (TTA) of the juice blend was between 0.112 for jackfruit juice and 0.08 for papaya juice respectively. Combination E showed highest TTA followed by combination A compared to other 3 combinations.

DPPH radical scavenging activity determination, Total phenolic content (TPC), Flavonoids content, Ascorbic acid content (AA)

For jackfruit and papaya combination the DPPH radical scavenging activity determination, total phenolic content, flavonoids content and ascorbic acid content are as follows:

Sr. No.	Sample	Combination (Jackfruit:Papaya) (ml)	DPPH ($\mu\text{g/ml}$)	TPC ($\mu\text{g/ml}$)	Flavonoids content (mg QE/100g)	Ascorbic acid content (mg/100ml)
1.	A	70:10	16.6687 $\pm$ 0.01	57.22588 $\pm$ 0.18	25.625 $\pm$ 0.05	6.872852 $\pm$ 0.14
2.	B	60:20	17.0952 $\pm$ 0.03	58.42105 $\pm$ 0.10	55.15625 $\pm$ 0.04	3.436426 $\pm$ 0.07
3.	C	50:30	16.9345 $\pm$ 0.00	59.42982 $\pm$ 0.03	98.75 $\pm$ 0.16	3.436426 $\pm$ 0.07
4.	D	40:40	17.0148 $\pm$ 0.00	59.27632 $\pm$ 0.04	166.09375 $\pm$ 0.08	5.154639 $\pm$ 0.07
5.	E	30:50	16.47095 $\pm$ 0.03	58.85965 $\pm$ 0.03	57.65625 $\pm$ 0.03	3.436426 $\pm$ 0.07
6.	F	80	16.71199 $\pm$ 0.01	57.91667 $\pm$ 0.17	58.28125 $\pm$ 0.03	3.436426 $\pm$ 0.07
7.	G	80	16.12485 $\pm$ 0.01	61.76535 $\pm$ 0.02	54.6875 $\pm$ 0.01	3.436426 $\pm$ 0.07

Table 6: DPPH activity, TPC, Flavonoid content and AA of jackfruit and papaya combination

The DPPH radical scavenging activity for jackfruit juice was 16.71199 and 16.12485 for papaya juice. The combination B showed highest antioxidant activity i.e. 17.0952 compared to other 4 combinations. In combination B 60 ml was jackfruit juice and 20 ml was papaya juice where it showed highest antioxidant activity. Because both the pure juice has antioxidant activity so, combination B showed highest antioxidant activity. The total phenolic content for jackfruit juice was 57.91667 and 61.76535 for papaya juice. Combination C and D showed highest TPC content compared to other 3 combinations. But pure juice of papaya showed higher TPC. The lowest TPC was recorded in combination A. The TPC of the juice blends were lower than pure papaya juice. The reduction in TPC value for the blend samples may be due to interaction between the phenolic compounds. The flavonoids content for jackfruit juice was 58.28125 and 54.6875 for papaya juice. The combination A showed highest flavonoids content followed by combination C. The lowest flavonoids content was found out in combination A. The combination D has equal concentration of jackfruit and papaya juice so it showed highest flavonoids content. But in combination A the concentration of jackfruit juice was high as compared to papaya juice. Both the juice has flavonoid present in them so, the flavonoids content was high in combination D followed by combination C. The ascorbic acid content (AA) for jackfruit juice was 3.436426 and 3.436426 for papaya juice. The combination A showed highest ascorbic acid i.e. 6.872852 which has 70 ml jackfruit juice and 10 ml papaya juice. Both the pure juices has ascorbic acid content present in them.

Sensory attributes of the juice blends

The overall acceptability for pomegranate juice was 7.06 and 6.73 for jamun juice. Combination A of pomegranate and jamun blend showed highest overall acceptability compared to other 4 combinations. The combination A has 70 ml pomegranate juice and 10 ml jamun juice. The taste, clarity, color, after taste and mouth feel of combination A was also very well accepted by the sensory members. The overall acceptability for jackfruit juice was 5.933333 and 6.066667 for papaya juice. Combination B of jackfruit and papaya blend showed highest overall acceptability compared to other 4 combinations. The combination B has 60 ml pomegranate juice and 20 ml jamun juice. The taste, clarity, color, after taste and mouth feel of combination B was also very well accepted by the sensory members.

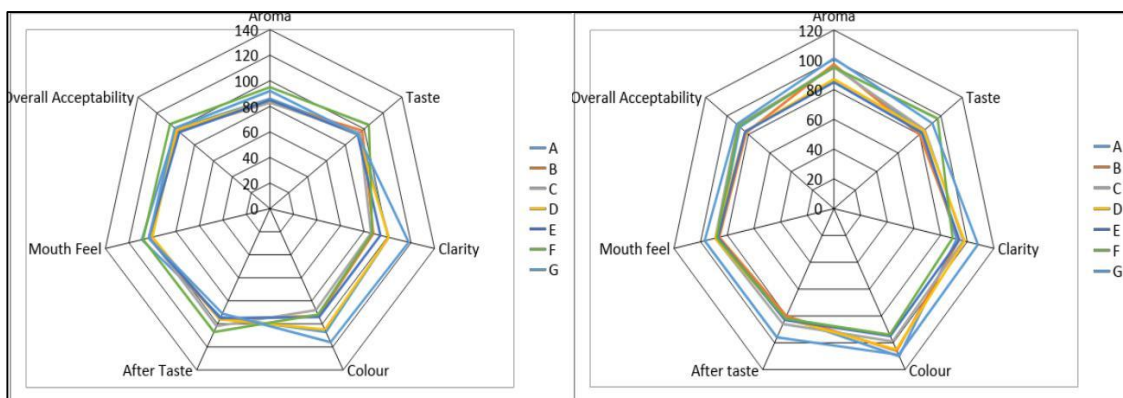


Figure 1: Pomegranate-jamun sensory chart Figure 2: Jackfruit and papaya sensory chart

Anthocyanin content, effect of pH on color stability and effect of stabilizers

Further anthocyanin content, effect of pH on color stability and effect of stabilizers on optimized combination A of pomegranate and jamun blend was studied which are as follows:

Sr. No.	pH values	Total anthocyanin content (mg/L)
1.	2.5	3.8240± 0.6
2.	4.0	3.5902± 0.4
3.	6.0	2.6885± 0.2
4.	8.0	2.5883±0.1

Table 7: Anthocyanin content in pomegranate-jamun combination

When the pH of the juice blend increased, the anthocyanin content decreased as shown in the table. The anthocyanin contents at pH 2.5 and 4.0 were higher than at pH 6.0 and 8.0. At pH 2.5 and 4.0 there was not a significant difference in the anthocyanin content. Similarly, at pH 6.0 and 8.0 there was not a significant difference in the anthocyanin content. Above result shows that more acidic juice blend of pomegranate-jamun (lower pH) will have higher anthocyanin content than less acidic pomegranate-jamun blend (higher pH). These differences likely arise because structurally anthocyanin is more stable under acidic conditions than neutral or alkaline conditions (Markakis 1982; Bae and Suh 2007).

The optimized pomegranate-jamun combination A was further studied for effects of stabilizers with different stabilizers and different concentrations. The stabilizers used were commercial pectin, Carboxymethyl cellulose and extracted pectin from pomegranate peel. The concentrations were 0.1%, 0.3% and 0.5% and one sample was without additives. The turbidity and viscosity was studied at different days for one week which are as follows:

The results for the viscosity of the pomegranate-jamun blend are shown in the above table. The viscosity of pomegranate-jamun was affected by storage period, mass fraction of the stabilizers and type of stabilizer. As the mass fraction of the stabilizers increased, the viscosity of the pomegranate-jamun blend also increased. As the storage time increased, the viscosity decreased. But in the sample 9 the viscosity increased on storage period and remained constant and in sample 6 it increased and decreased again. In sample 6 after 1st day the viscosity increased on 4th day and decreased on 6th day. 0.5% CMC gave the highest viscosity followed by extracted pectin compared to other stabilizers. Sample 10 which were without addition of stabilizers showed low viscosity. The turbidity of the pomegranate-jamun blend was studied during storage at 4°C for 1 week. The storage period, mass fraction of the stabilizers and type of stabilizers affected the turbidity. The turbidity increased on the storage period of pomegranate-jamun blend. The highest turbidity was

observed in sample 6 which has 0.5% CMC. The lowest turbidity was observed in sample 9 which contain 0.5% extracted pectin. The increased in turbidity may be due to protein, pectin and polyphenol.

4. CONCLUSION

The addition of jamun juice to pomegranate juice and papaya juice to jackfruit juice contributes to acceptable juice blends in terms of taste; colour and mouth feel by the sensory panelists. The combination A (70:10) of pomegranate and jamun showed highest overall acceptability compared to other 4 blends. The antioxidant activity was highest in combination A of pomegranate-jamun blend but the flavonoid content was highest in combination D (40:40) which has equal concentration of pomegranate and jamun juice. The TPC content was highest in both the pure juices followed by combination A. The combination B (60:20) of jackfruit and papaya showed highest overall acceptability compared to other 4 blends. The antioxidant was highest in combination B of jackfruit and papaya blend but the flavonoid content was highest in combination D (40:40) which has equal concentration of juice. The TPC content was higher in pure papaya juice followed by combination C. Further, anthocyanin content, effect of pH on colour stability and effect of stabilizers on optimized combination A of pomegranate-jamun blend was studied.

Sample Number	1st day reading		4th day reading		6th day reading	
	Turbidity (500 NTU-STD)	Viscosity	Turbidity (500 NTU-STD)	Viscosity	Turbidity (500 NTU- STD)	Viscosity
1.	430	5.2	433	5.0	435	4.98
2.	437	5.7	436	5.28	437	4.98
3.	433	8.1	434	7.74	434	7.07
4.	428	5.4	427	5.04	429	5.00
5.	436	8.7	438	5.08	440	4.97
6.	462	5.8	463	5.1	468	5.0
7.	430	5.2	433	5.04	436	4.96
8.	432	7.3	431	6.92	435	6..66
9.	418	10	420	11	423	11
10.	455	4.32	456	4.12	458	4.02

Table 8: Determination of turbidity and viscosity of pomegranate-jamun combination

5. CONFLICT OF INTEREST

The authors declare no conflict of interest relevant to this article

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Acrylamide Reduction In Fried Products Using Oil Soluble Vitamins

A. RADHAKRISHNAN, J. D'SOUZA, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India

ABSTRACT: Acrylamide is the major concern in fried and baked foods. Acrylamide is formed when starchy foods are cooked at high temperature (120°C). The major pathway leading to acrylamide formation is a part of the maillard reaction with asparagine and reducing sugars. The reduction of acrylamide was carried out by fortifying the oil with vitamin A, D₂, E and E acetate. The normal French fries without fortification of the oil were found to have 8.045±0.00 ppm. The French fries samples were fried at the maximum permitted concentration of vitamins in oil according to food safety standards authority of India (FSSAI). The analysis of acrylamide was carried out by HPLC method. The acrylamide content in vitamin A, D₂, E and E acetate fortified French fries samples were found to be 0.000±0.00 ppm, 0.000±0.00 ppm, 0.029±0.05 ppm and 0.020±0.03 ppm respectively. The vitamin fortified French fries acrylamide reduction was found to be 100%, 100%, 99.6% and 99.7 % in vitamin A, D₂, E and E acetate respectively. No samples were rejected by the untrained panelists.

KEYWORDS: Vitamin A, vitamin D₂, vitamin E, Vitamin E acetate, Acrylamide, fortification

***Corresponding Author: Mr. Ashish Dabade**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed To Be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Acrylamide (C₃H₅NO) of molecular weight 71.08kDa is water soluble non volatile colourless crystalline solid. The International Agency for Research on Cancer (IARC) defined acrylamide as a potential carcinogen to human based on its carcinogenicity in rodents. Acrylamide formation was first reported by the Swedish National food administration 2002 in foods[1].

Oil heated for frying at temperature above 120°C can generate potentially cancer causing agents or carcinogens like heterocyclic amines, polycyclic aromatic hydrocarbons, aldehydes, acrolein and acrylamide [2].

Acrylamide are majorly found in fried potatoes and potato chips, also in foods with high starch concentration when cooked at high temperature. Long-term studies in rats and mice supported a dose–exposure relation between acrylamide and risk of cancer of the lung, mammary gland, thyroid, oral cavity, and intestinal and reproductive tract[3].

Antioxidants have a major influence to the maillard reaction, which results in acrylamide formation. Antioxidants present in Rosemary extracts, bamboo leaves and green tea extracts have found to effectively reduce the acrylamide activity in heated foods. Antioxidants have proved to block the oxidation of acrolein formation and hence reduce acrylamide content[1].

Free radicals are formed during the maillard reaction. Free radicals are those which consist of one or more unpaired electron and are highly reactive. Therefore this single unpaired electron is highly active to steal an electron from a neighboring atom by making itself stable and forming the neighbor atom a free radical, and hence this leads to a chain reaction ultimately damaging tissues.[4]. Thereby antioxidant plays a crucial role in stabilizing the free radical and hence reduces the tissue and cell damage that is caused due free radical chain reaction[5].

Vitamins also act as antioxidants in food, also a good source of nourishment. Vitamin A, D and E are good source of antioxidants. α -Tocopherol donates its phenolic hydrogen atom to a peroxy radical and converts it to hydro peroxide. The tocopheroxyl radical that is formed is sufficiently stable to be unable to continue the chain. Hence vitamin E acts as an antioxidant [6].

The antioxidant activity of vitamin A arises in different ways. It can act as chain breaking antioxidant by combining with peroxy radicals, before these radicals can propagate peroxidation in the lipid phase of the cell and generate hydro peroxides. Hence they act as an efficient radical scavenger[7]. Also vitamin D acts as a membrane antioxidant and helps to stabilize the highly reactive free radical[8].

2. MATERIALS AND METHODS

2.1.Reagents and chemicals

15 kg non fortified sunflower oil gift sample was provided by AAK Kamani private limited. Amul happy treats French fries was purchased from local market. Acrylamide 3X crystal for molecular biology purchased from SIGMA. Vitamin E, E acetate, vitamin A purchased from HIMEDIA and Vitamin D₂ was purchased from SRL. N-Hexane and HPLC Water for chromatography purchased from MERCK. Carrez 1 – Potassium hexaferrocyanide and Carrez 2 – Zinc acetate purchased from SRL.

2.2.Method

2.2.1.Vitamin A fortification

25 International Unit (IU)/g of Vitamin A was fortified in 2 literes of non – fortified sunflower oil. The frozen French fries were fried in this oil for 3minutes at 175°C in an electric Deep fryer (Make-Skyline Deep fryer)

2.2.2.Vitamin D₂ Fortification

4.5 International Unit (IU)/g of Vitamin D₂ was fortified in 2 literes of non – fortified sunflower oil. The frozen French fries were fried in this oil for 3minutes at 175°C in an electric Deep fryer (Make- Skyline Deep fryer)

2.2.3.Vitamin E and E acetate

50 International Unit (IU) of Vitamin E and E acetate was fortified in 2 literes of non – fortified sunflower oil. The frozen French fries were fried in this oil for 3minutes at 175°C in an electric Deep fryer (Make- Skyline Deep fryer)

2.2.4. Sample preparation

Sample preparation was done as per the method mentioned by Zokaei et. al; (2016). French fries samples were homogenized well. 5 grams of homogenized sample were weighed. Fat separation was performed to the weighed samples by addition of 7.5ml n-Hexane and 17.5ml of water and centrifuged for 4000 rpm for 5mins. Carrez 1 and Carrez 2 of 1.25ml each were added to the fat separated sample to precipitate the carbohydrates and proteins and centrifuged at 4000 rpm for 5 min. The supernatant was collected after filtration using millipore filter paper. [9]

2.2.5. Acrylamide analysis by HPLC

The acrylamide analysis was carried out by HPLC method as mentioned in Gokmen et. al; (2004). Sample analysis was done using water as the mobile phase at a flow rate of 1ml/minute. A C18 5um column was used for analysis. Readings were compared with standard readings of known concentrations to obtain results to conclude if there has been any reduction in the formation of acrylamide or not.[10]

2.2.6. Statistical Analysis

Reduction percentage of acrylamide was calculated by

$$\frac{(\text{Control acrylamide level} - \text{After fortification acrylamide level}) \times 10}{\text{Control acrylamide level}}$$

3. RESULTS AND DISCUSSION

Effect of fortification of vitamins on acrylamide formation –

According to the HPLC graph readings obtained, control sample that is the sample fried in non-fortified oil showed a peak at RT 3.11 with the peak corresponding to the amount of acrylamide equal to 8.045ppm. This was compared with the graph of standard of 50ppm acrylamide

In this study, four vitamins were examined for their effect to reduce acrylamide generation in fried French fries. Reduction of acrylamide was found up to 100%±0% in Vitamin A and Vitamin D₂ fortified fried French fries (Fig.2). Fortification provided by Vitamin E and Vitamin E acetate were showing 99.6%±0% and 99.7%±0% reduction (Fig.2). The complete inhibition of acrylamide synthesis was observed in vitamin A and D₂ fortified oil fried product (Table.1).

Antioxidants are one of the crucial factors to mitigate the acrylamide formation in foods. Thereby antioxidant plays a crucial role in stabilizing the free radical and hence reduces the tissue and cell damage that is caused due free radical chain reaction.

Vitamins A, D₂, Vitamin E and Vitamin E acetate have potential antioxidants present which reduce the acrylamide reduction in fried products. Also nutritional enhancement with the help of vitamins can also be obtained by fortifying the oil with vitamins. Hence vitamins play an important role in the acrylamide reduction in fried foods.

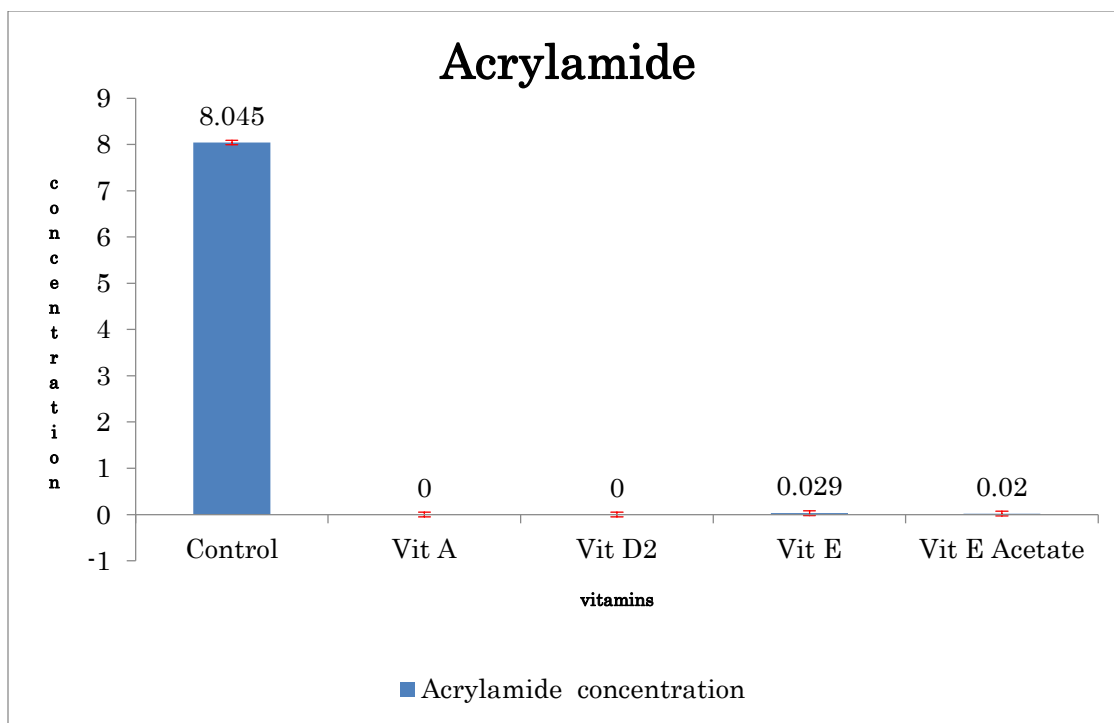


Figure 1: Acrylamide concentration in vitamin fortified oil fried product. $n=3$

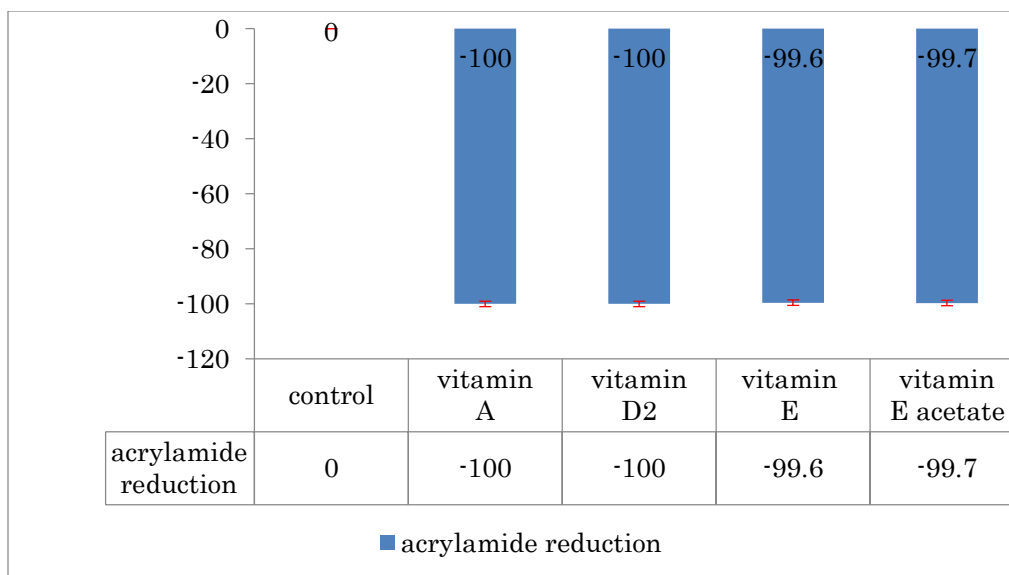


Figure 2: Percentage reduction of acrylamide in Vitamin Fortified French fries ($n=3$)

4. DISCUSSION

Deep fried French fries Fortified in Vitamin A, D₂, E and E acetate oil have been analysed and the acrylamide reduction data suggests that Vitamin A, D₂, E and E acetate have a good potential not only as nutritional quality in food processing but also can be used for reduction of carcinogenic components like acrylamide. The vitamins which are fortified have not only served the purpose of

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reduction of acrylamide but also have helped in reducing the vitamin deficiency and able to provide better nourishment to the ones needed. Hence along with consumption of vitamin nourished fried food it cancels the fear of acrylamide formation in fried products and French fries.

5. CONCLUSION

I am thankful to School of Biotechnology and Bioinformatics, D. Y. Patil Deemed to be University for allowing me to work on this project at the esteemed Institute.

I am thankful to AAK Kamani Pvt. Ltd. Khopoli, Maharashtra India for providing us with the necessary ingredients for supporting our work.

6. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Hydrocolloid Based Edible Coatings to Reduce Acrylamide in Fried Products

S. IYER, A. PATKAR, S. KUDALE, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: Acrylamide is the major concern in fried and baked foods. The major pathway leading to acrylamide formation is a part of the maillard reaction with asparagine and reducing sugars. Acrylamide is a potential carcinogen in foods. The reduction of acrylamide was carried out by hydrocolloids like Methylcellulose (MC) and carboxymethylcellulose (CMC). The French fries samples were coated with 0.5%, 1%, 1.5% and 2% CMC and 0.5%, 1% and 1.5% MC individually. The acrylamide analysis was carried out by HPLC method. The control samples of French fries without coating had 8.045 ppm acrylamide. The acrylamide content in 0.5%, 1%, 1.5% and 2% CMC coated French fries samples were found to be 1.212 ± 1.051 , 0.000 ± 0.00 , 0.000 ± 0.00 and 0.000 ± 0.00 respectively. The acrylamide content in 0.5%, 1% and 1.5% MC coated French fries samples was found to be 0.543 ± 0.94 , 0.000 ± 0.00 and 0.000 ± 0.00 respectively. The carboxymethylcellulose coated acrylamide reduction was found to be 84.93%, 100%, 100% and 100% in 0.5%, 1%, 1.5% and 2% CMC respectively. The methylcellulose coated acrylamide reduction was found to be 93.25%, 100% and 100% in 0.5%, 1% and 1.5% MC respectively. No samples were rejected by the untrained panelists.

KEYWORDS: Carboxymethylcellulose, Methylcellulose, Acrylamide, Maillard reaction, Edible coating, French fries

***Corresponding Author: Mr. Ashish Dabade**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed To Be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu

1. INTRODUCTION

Acrylamide (C_3H_5NO) is a water soluble colourless crystalline solid with molecular weight of 71.08kDa. It is non-volatile. The International Agency for Research on Cancer (IARC) in 1994 classified acrylamide (2-propenamide) based on its carcinogenicity in rodents (Group 2A), as a potential carcinogen to humans [1].

Oil heated for frying at temperature above $120^\circ C$ can generate potentially cancer causing agents or carcinogens like heterocyclic amines, polycyclic aromatic hydrocarbons, aldehydes, acrolein and acrylamide [2].

High concentrations of acrylamide are found in fried potatoes and potato chips and, more generally, in starch-containing foods cooked at high temperatures. Long-term studies in rats and mice supported a dose–exposure relation between acrylamide and risk of cancer of the lung, mammary gland, thyroid, oral cavity, and intestinal and reproductive tract [3].

Water activity in food plays a major role in reducing acrylamide formation. Acrylamide forms in

food only when the water activity is below 0.8, whereas the acrylamide formation is high at water activity of 0.4 and below. However, the removal of acrylamide from heated foods such as biscuits and potato chips increases with the increase of water activity [1].

All hydrocolloids interact with water, reducing its diffusion and stabilizing its presence. CMC can be used for its water-holding capacity as it is high even at low viscosity; particularly when used as the Ca^{2+} salt. Thus, it is used for reducing fat uptake into fried foods. Methyl cellulose (or methylcellulose) enables a firmer texture to be achieved during pre-cooking, frying and during the final re-heat for serving. It has good moisture holding capacity and thus can be used in various fried products like boondi's batter preparation to achieve desired consistency [4].

Methyl cellulose (or methylcellulose) enables a firmer texture to be achieved during pre-cooking, frying and during the final re-heat for serving. The binding properties and gel strength provide desired ingredient cohesion and retention of the designed product form. MC can be used for the prevention of dehydration of frozen food. The use of hydrocolloids such as pectin, alginic acid and xanthan gum in the reduction of acrylamide have shown significant reduction ($p < 0.05$) of $>50\%$ by pectin and alginic acid and 20% by xanthan gum [5].

Hence, in the present study efforts have been carried out regarding application of edible surface coating for french fries in order to reduce acrylamide synthesis during the process of frying.

2. MATERIALS AND METHODS

2.1. Materials

15kg non-fortified Sunflower oil gift sample was provided by AakKamaniTM Private Limited, Khopoli, Maharashtra, India. Amul Happy TreatsTM french fries was purchased from local market, Mumbai, Maharashtra, India. Carboxymethyl cellulose purchased from SD fine-chem limited. Acrylamide purchased from SIGMA. Methyl cellulose purchased from HIMEDIA.

2.2. Methods

2.2.1. Preparation of coating solution

Coating suspensions were prepared with varying concentrations of CMC and MC using distilled water. These suspensions were stirred for 10 mins at 60°C . After cooling to room temperature, these solutions were used for coating purpose.

2.2.2. Application of surface coating to French fries samples

The frozen French fries for samples were dipped in different coating solutions for 10 seconds at ambient temperature conditions.

2.2.3. Frying of coated French fries samples

Frying was carried out in preheated sunflower oil at 175°C for 3 minutes in an electric deep fryer (Skyline deep fryer).

2.2.4. Sample preparation

Sample preparation was done as per the method mentioned by Zokaei et. al; (2016) Prepared French fries samples were pulverized well. 5 grams of each sample was weighed in a centrifuge tube. In the next step, fat separation was performed using 7.5 mL of n-hexane, and then 17.5 mL distilled water was added and centrifuged at 4000 rpm for 5 min. Then the upper aqueous phase was separated, and 1.25 mL of Carrez solution I (potassium hexaferrocyanide) and 1.25 mL of Carrez solution II (zinc acetate) were added to precipitate the soluble carbohydrate and protein. This sample was thoroughly agitated and then centrifuged for 5 min at 4000 rpm. Supernatant was filtered with whatman paper and collected for analysis.[6]

2.2.6.Acrylamide analysis by HPLC

The acrylamide analysis was carried out by HPLC method as mentioned in Gokmen et. al; (2004). The samples were filtered using a millipore filter with pore size 0.45um and diameter 0.22um. Sample analysis was carried out by using water as the mobile phase with a flow rate 1ml/minute. C18, 5um column was used for analysis. Readings were compared with standard readings of known concentrations of acrylamide.[7]

2.2.6.Statistical and comparative analysis

Statistical analyses of the samples were carried out by SPSS Software module. One way ANOVA compare means were analysed. The significant difference study was carried out with homogenized subset comparison by Duncan method. The reduction of acrylamide level calculated by formula: Reduction in percentage =

$$\frac{(\text{Control acrylamide level} - \text{After coating acrylamide level}) \times 100}{\text{Control acrylamide level}}$$

3. RESULTS AND DISCUSSION AND DISCUSSION

3.1.Effect of hydrocolloids on acrylamide formation

According to the HPLC graph readings obtained, control sample that is the sample without any coating showed a peak at RT 3.11 with the peak corresponding to the amount of acrylamide equal to 8.045ppm. This was compared with the graph of standard of 50ppm acrylamide.

In this study, two hydrocolloids were examined for their effect to reduce acrylamide generation in fried French fries. Reduction of acrylamide was found upto 100%±0% in 2% CMC and 1.5% MC coated fried frenchfries (Fig.2). The acrylamide concentration shown by coated samples were significantly different ($p>0.05$). Treatment provided by 1% CMC and 0.5% MC were showing significantly different results than control (Table.1). The application of 1%, 1.5% and 2% CMC results were not significantly different ($p<0.05$). Hence 1% CMC coating effect on French fries was considered as optimized concentration of CMC to reduce acrylamide completely (Table.1). The application of 0.5%, 1% and 1.5% MC results were not significantly different ($p<0.05$). Hence 0.5% MC coating effect on French fries was considered as optimized concentration of MC to reduce acrylamide completely (Table.1).

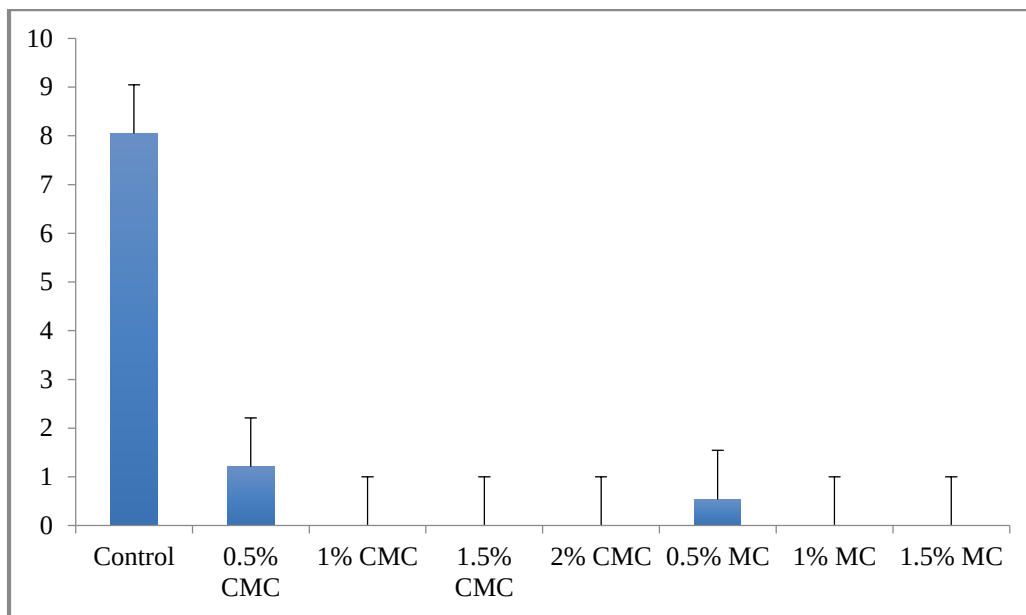


Figure 1: Acrylamide concentration in Hydrocolloid coated fried French fries

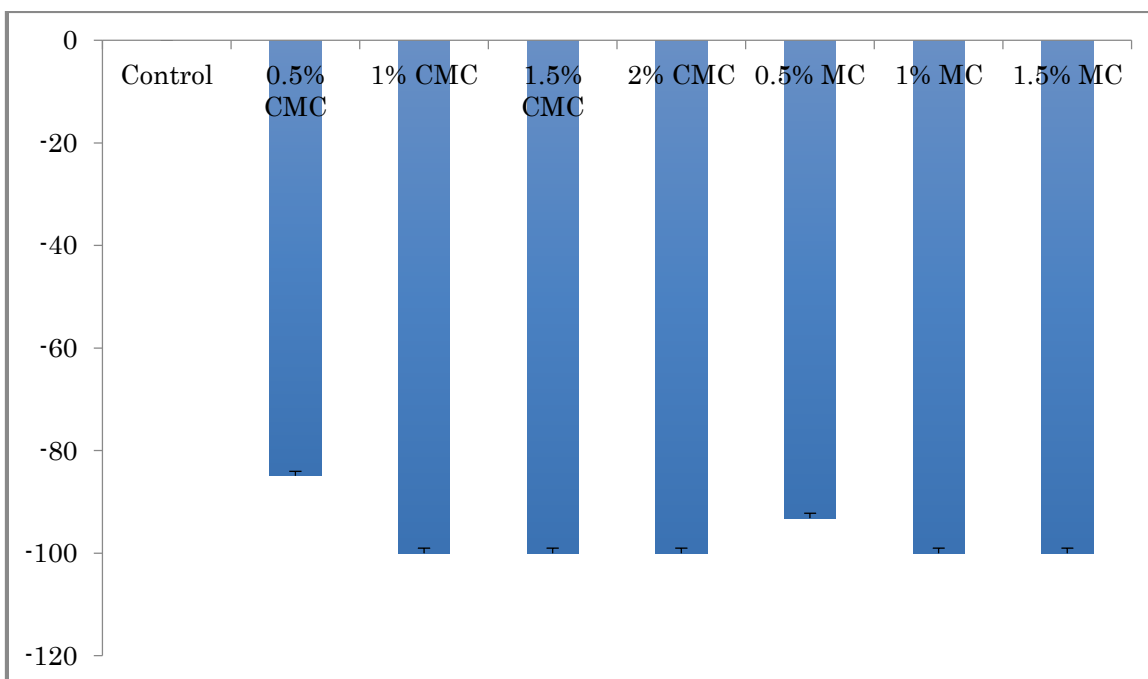


Figure 2: Percentage reduction of acrylamide in Hydrocolloid coated French fries

Water activity is a major factor to control acrylamide formation in fried foods[1]. The water activity of French fries can be controlled with 1% of CMC and 0.5% of MC to reduce acrylamide concentration. This postulate of water activity not observed in this study as it is already mentioned earlier that increasing the concentration of hydrocolloids can maintain water activity at a particular level. However, the reduction of oil absorption was also proved with hydrocolloids. Hence the reduction of oil content, as well as maintenance of water activity synergistically might reduce

acrylamide synthesis. Apart from water activity, the modification in the surface structure of French fries might have influence on acrylamide reduction.[8]

4. CONCLUSION

Deep fried French fries coated with 1% CMC and 0.5% MC Acrylamide reduction data might suggests that the CMC and MC have good potential not only as thickeners and stabilizers in food processing but also can be used for reduction of carcinogenic components like acrylamide. The quality enhancement of fried products and snacks can be possible with CMC and MC at very low cost modification.

Hydrocolloid coating concentration	Acrylamide concentration in CMC coated fried French fries	Acrylamide concentration in MC coated fried French fries
CONTROL	8.045±0.000 ^a	8.045±0.000 ^a
0.5%	1.212±1.051 ^b	0.543±0.940 ^b
1%	0.000±0.000 ^c	0.000±0.000 ^b
1.5%	0.000±0.000 ^c	0.000±0.000 ^b
2%	0.000±0.000 ^c	NA

Table1: Acrylamide Concentration in Hydrocolloid Coated Fried French Fries

5. ACKNOWLEDGEMENT

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6. CONFLICT OF INTEREST

We hereby also confirm that the work does not have any conflict of interest issues.

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Development of Protein Based Emulsifier from Fermented Flowers of *Madhuca longifolia*

K. KUMAR, P. RELEKAR, K. SARVATE, A. GUPTE *, A. DABADE*

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

ABSTRACT: *Madhuca longifolia* has got many Pharmaceutical, nutritional, biological and anti-oxidant properties as well. Mahua is rich in Sugar, Vitamin, Protein, Alkaloids, and Phenolic components and it also has Ethno medical properties like Anti-bacterial, Anti-cancer, Anti-ulcer, Anti-hyperglycemic, and Analgesic activity. The study investigated that the extraction of protein from *Madhuca longifolia* can be done by three different methods. And out of which, TCA-Acetone method showed the best result for protein extraction. The total amount of protein produced in TCA-Acetone method was higher than alkaline method. *Madhuca longifolia* flower TCA- Acetone treated protein sample had the highest emulsion activity with the value 0.903 (Abs500) at 5%. Whereas, at 2% concentration, it has the highest stability than 5%. Foaming capacity and stability of *Madhuca longifolia* flower TCA-Acetone treated protein sample increased from 1 to 5% and no foaming capacity and stability were observed in case of the Alkaline treated sample but they can be used as an Antifoaming agent in future. The results also showed that water holding capacity and oil holding capacity of *Madhuca longifolia* flower is more than soy lecithin. Mahua has a got huge potential in future in the food industry as biscuits, candy, wine, sugar syrup, antifoaming agent. They can be also used to make jam, laddu, and pickles.

KEYWORDS: *Madhuca longifolia*, emulsion, foaming, stability, Soylecithin, water holding capacity.

***Corresponding Author: Mr. Ashish Dabade/ Dr Arpita Gupte**

School of Biotechnology and Bioinformatics, D.Y. Patil Deemed to be University, Food Science and Technology, CBD Belapur, Navi Mumbai, India.

Email Address: ashish.dabade@dypatil.edu / arpita.gupte@dypatil.edu

1. INTRODUCTION

Plants are a rich source of drugs and a vast reservoir of chemicals and thus it has great importance in Pharmaceutical industries. The drug used in Ayurveda and Unani system are from the plant source. World health organization these day is focusing attention to encourage the use of herbal medicines. Herbal medicine can be defined as those products which are simply derived from any part of a plant. The knowledge of traditional medicine put light on the discovery of new and potent medicines [1].

Mahua has got many Pharmaceutical, nutritional, biological and anti-oxidant properties as well. The study shows that Mahua is rich in Sugar, Vitamin, Protein, Alkaloids, and Phenolic components and it also has Ethno medical properties like Anti-bacterial, Anti-cancer, Hepatoprotective, Anti-ulcer, Anti-hyperglycemic, and Analgesic activity.[2]

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Madhuca longifolia is highly regarded as a universal panacea in the Ayurvedic medicine. Mahua is a large evergreen tree distributed in India, Sri Lanka and Nepal [3]. Mahua is a deciduous tree belonging to the family *Sapotaceae*. It has a significant place in tribal culture. The tree is 10-15 m tall with spreading, dense, round and shady canopy. Mahua whose genus is *Madhuca* is a multipurpose tree with different species as *Madhuca longifolia*, *Madhuca latifolia*, and *Madhuca butyracca*. Mahua is a large shady canopy like tree especially found in central, south and north states of Jharkhand, Orissa, Madhya Pradesh, Bihar, Kerala and some part of Maharashtra. The tree has got economic, nutritional and pharmaceutical value because of its flowers, fruits, oil, bark, seedcake, and timber [4]

Madhuca longifolia seed cake is used for feed application to poultry and other animals. Being rich in protein, they are also considered as ideal for the food supplement. They can be used as an Energy source, production of enzymes, production of Mushroom, production of Antibiotics and Bio-pesticides, Preparation of protein hydrolysable [5].

One another important key aspects of seed cake are that they are relatively cheaper and easily available throughout the year. Oil cake is profitably utilized as biofertilizers, organic manure and biocide (insecticide and herbicide properties) in different crops. After detoxification, seed cake can be used as a concentrate feed for cattle and fish [6]

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Mahua longifolia seed, flowers, after the fermented flower and seed cake was obtained from a village- Manika, latehar district, Jharkhand state, and a sample was tightly packed and sealed in the polythene bag. Soy lecithin and sunflower oil were purchased from a local shop and all the chemicals purchased from SRL labs.

2.2. Methods

2.2.1. Proximate analysis

Proximate analysis of *Madhuca longifolia* after fermented flower in terms of moisture content, protein, fat, ash, total sugar, energy, carbohydrate was estimated [7]

2.2.2. Method to extract protein from *Madhuca longifolia* after fermented flower

Protein yield comparison for different Extraction: Method B- The protein was extracted by alkaline extraction[8]. Method A- The protein was extracted by TCA [9] Method C –TCA acetone without mercaptoethanol[8].

2.2.3. Methods of estimation of *Madhuca longifolia* after fermented extracted protein sample

Protein estimation was carried out by using Kjeldhal method and nitrogen (%) obtained was then multiplied by the factor of 5.95 to obtain % of crude protein. Carbohydrate content was determined by subtracting the sum of protein, fat, ash and moisture content from 100.

Protein estimation was done by Kjeldahl method; it gives the approximate value of protein[10].

2.2.4.Emulsion preparation

Oil in water type emulsion was prepared for soy lecithin and *Madhuca longifolia* after fermented flowers.[11]

2.2.6.Emulsion activity and stability

Emulsifying activity and stability of after fermented flower were determined by using along with some modification [12].

Emulsion stability Index was calculated using the following equation

$$\text{Emulsion stability} = A_0 \times t / A$$

A₀=Absorbance at time 0 min

A=change in absorbance during the time interval; t (10min)

2.2.7.Foaming capacity and foaming stability

Foaming capacity and Foaming stability were determined with some modification and their values were calculated by using the given formula.[13]

$$\text{FC\%} = (\text{volume after agitation} - \text{volume prior to agitation}) \times 100 / \text{volume prior to agitation}$$

The samples were kept at room temperature for 30 mint to estimate FS using following formula

$$\text{FS\%} = (\text{Residual foam volume}) / \text{total foam volume} \times 100$$

2.2.8.Water holding capacity

Water holding capacity were determined by method was used to determine water holding capacity along with some modification and their values were calculated by using the given formula.[14]

The amount of absorbed water was calculated by given formula

$$\text{Water (gm) absorbed} = \text{final weight} - \text{initial weight}$$

2.2.9.Oil holding capacity

Oil holding capacity was determined along with some modification and their values were calculated by using the given formula.[13]

$$\text{Oil (gm) absorbed} = \text{final weight} - \text{initial weight}$$

3. RESULTS AND DISCUSSION

Proximate analysis of *Madhuca longifolia* after fermented flower in terms of moisture content, protein, fat, ash, total sugar, energy, and carbohydrate was estimated (Table.1.)

All the three methods were used to extract the protein content and maximum yield

All the three methods were compared and out of which method B is considered the best method for protein extraction and these samples were further used for the emulsion and its functional properties. (Table 2)

Emulsion activity and stability

The emulsion activity (EA) and emulsion stability (ES) of Mahua after fermented flower protein for TCA-Acetone and Alkaline treated samples and control (soy lecithin) for different concentration

COMPOSITION	CONTENT (g per 100gms)
Moisture	9.3
Ash	7.8
Fat	2.3
Protein	14.4
Total sugar	4.1
Carbohydrate	64.0

Table 1: Proximate Composition of Madhuca Longifolia after a Fermented Flower

METHODS	PROTEIN YIELDS (%)
Method -A	14.04
Method -B	19.03
Method -C	11.07

Table 2: Comparison Analysis of Three Protein Extraction Methods

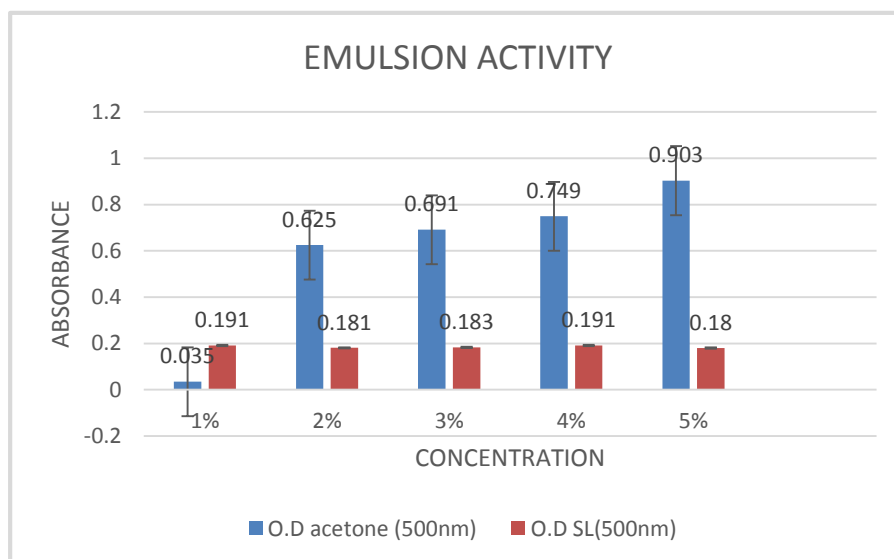


Figure 1: Represent the comparison between emulsion activity of Mahua TCA-Acetone treated protein sample and soy lecithin. The value was max at 5% i.e. 0.903 and min at 1% i.e. 0.035 for TCA-Acetone treated sample.

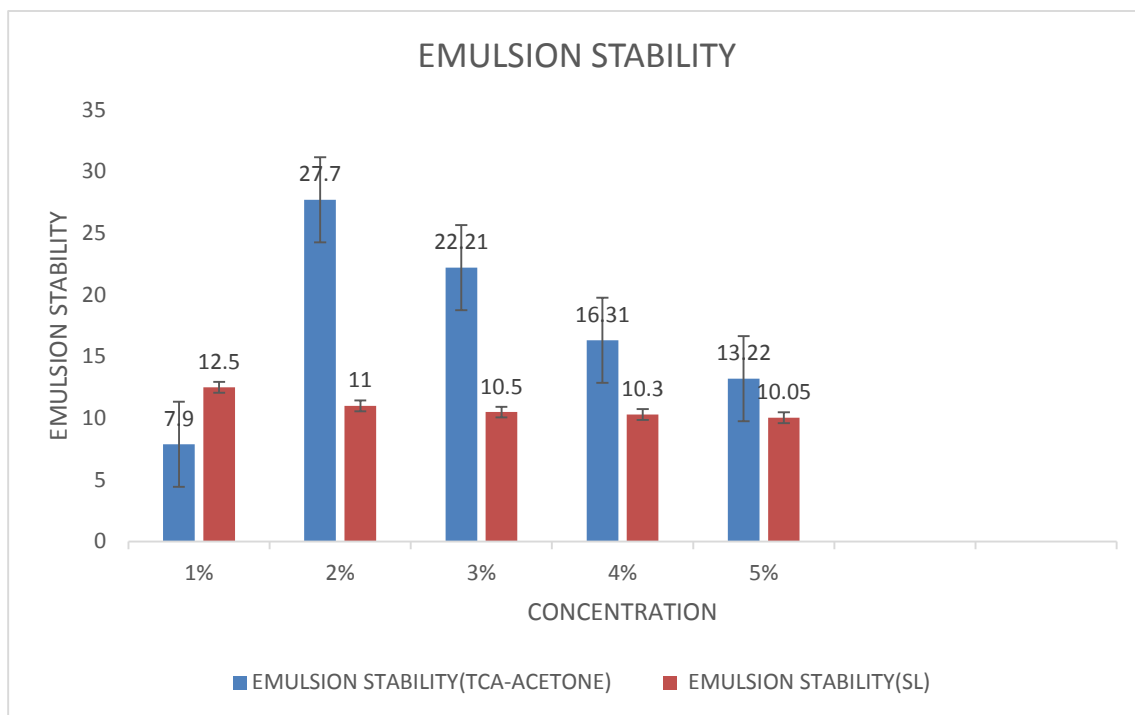


Figure 2: Represent the comparison between emulsion stability of Mahua TCA-Acetone treated protein sample and soy lecithin. Emulsion stability of TCA-Acetone is max at 2% and min at 1%

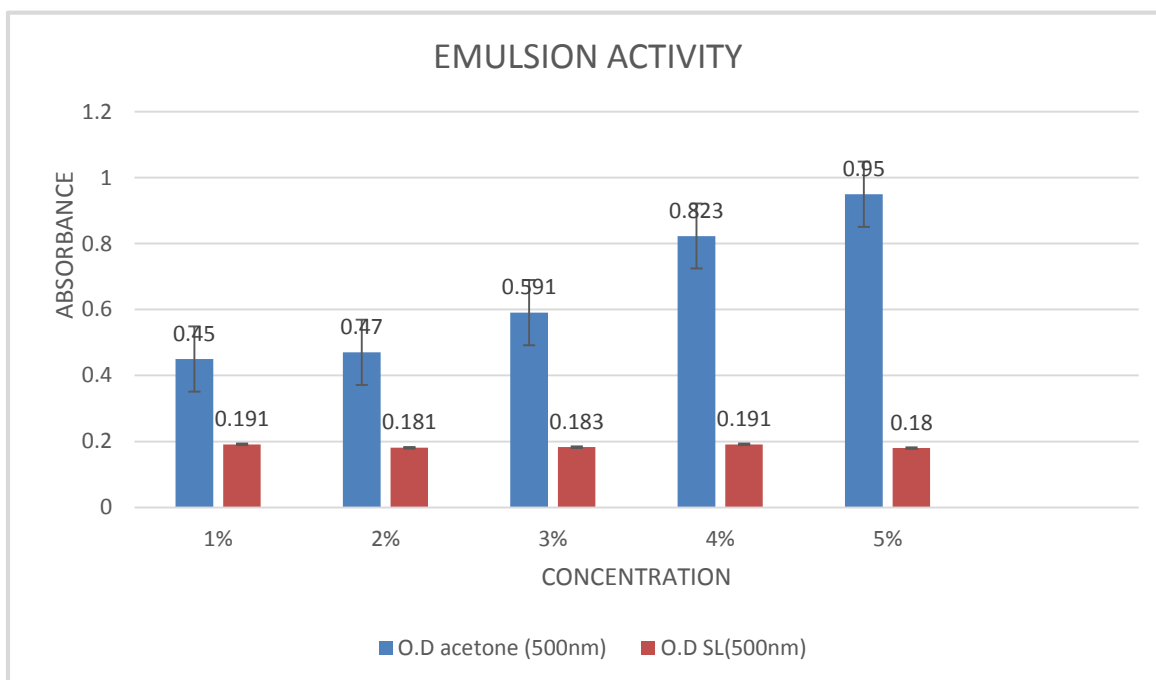


Figure 3: Represent the comparison between emulsion activity of Mahua Alkaline treated protein sample and soy lecithin. The emulsion activity of Alkaline treated sample was max at 5% i.e. 0.95 and min at 1% i.e. 0.45

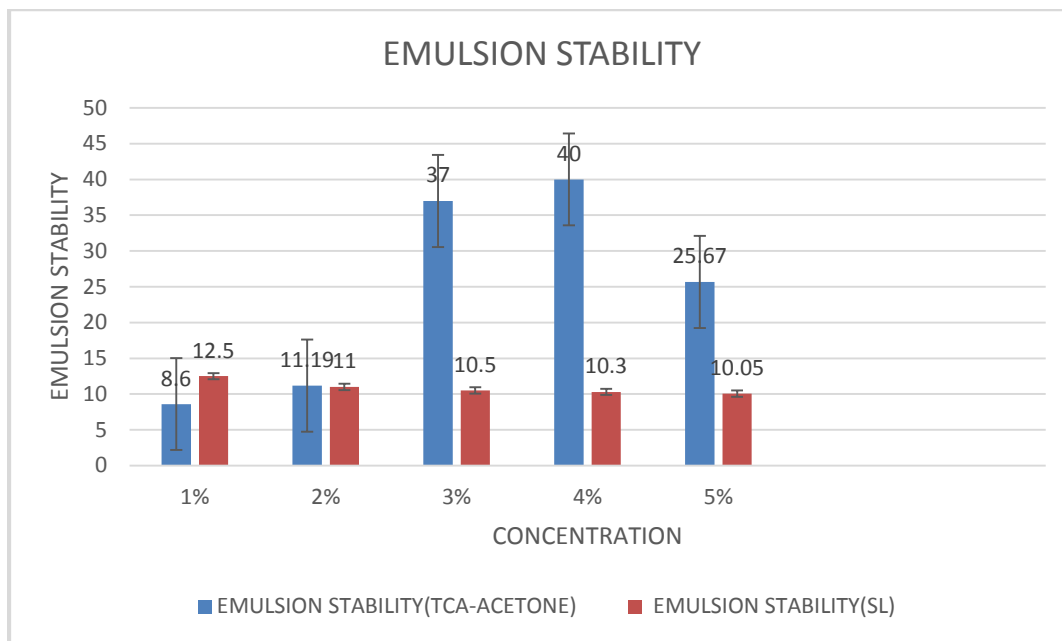


Figure 4: Represent the comparison between emulsion stability of Mahua Alkaline method flower treated protein sample and soy lecithin. The emulsion stability was found to be max at 4% i.e. 40 and min at 1% i.e. 8.6

Foaming capacity and foaming stability

The Foaming capacity (FC) and Foaming stability (FS) of Mahua after fermented flower protein for TCA-Acetone and Alkaline treated samples and control (soy lecithin) for different concentration. Madhuca longifolia after fermented flower TCA-Acetone protein sample has the foaming capacity as concentration increased from 1% to 5% with different value percentage. After stands for 30 min, the foaming stability of 1% is stable, whereas, it decreased as the concentration increases from 1% to 5%. And it was observed that Alkaline treated sample has no foaming capacity and foaming stability.

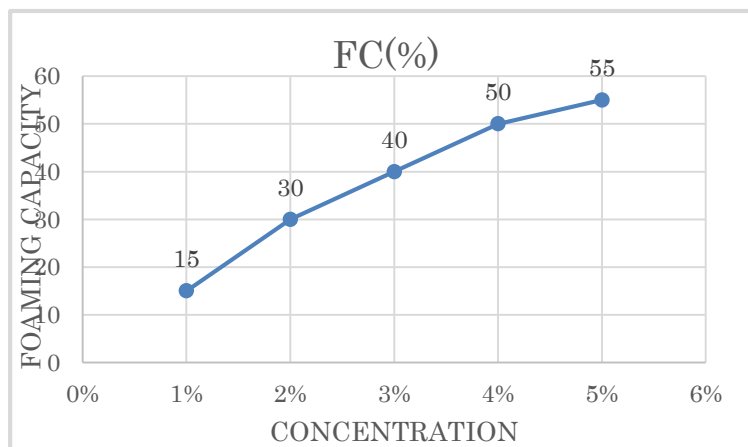


Figure 5: Effect of different concentration on foaming capacity of TCA-Acetone Mahua treated protein sample and the value of foaming capacity increases with the increase in the concentration.

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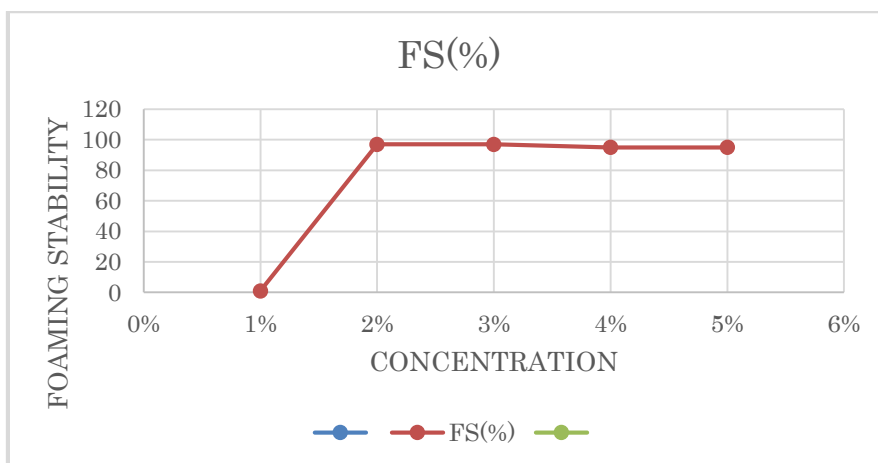


Figure 6: Effect of different concentration on foaming Stability of TCA-Acetone Mahua treated protein sample. The values of foaming stability first increases and then it becomes stable

Water holding capacity and oil holding capacity

The water holding capacity and oil holding a capacity of Mahua after fermented flowers TCA-Acetone treated protein is more than soy lecithin i.e. 2.99 and 2.03. (Table 3)

The water holding capacity and oil holding a capacity of Mahua after fermented flowers Alkaline treated protein sample.

The water holding capacity and oil holding the capacity of Mahua after fermented flowers Alkaline treated protein is more than soy lecithin i.e. 3.97 and 1.032. (Table 3)

Water holding capacity of flour with Alkaline treated Mahua flower has higher (4.544) then other combination. This protein sample was added to check the softness of chapatti. (Table 4)

Composition	TCA-Acetone treated Mahua sample	Alkaline treated Mahua sample	Soy lecithin
Water Holding Capacity(WHC)	4.03	3.97	0.79
Oil-Holding Capacity(OHC)	2.03	1.032	0.42

Table 3: Comparison of Alkaline Treated and Tca-Acetone Mahua Sample with Soy Lecithin

4. DISCUSSION

Madhuca longifolia is a great asset to food industry. It is mostly grown in the states of Bihar, Jharkhand, Orissa and some parts of Maharashtra. Mahua tree has got many applications in food industry and medical. It is used as a staple food, preparing traditional liquor “*hadiya*”, cake, biscuits, juice and wine. But in spite of numerous of applications of this traditional plant yet failed

to make its mark in the recent decades. In the present study it was used to evaluate the protein extraction yield from three different methods. Table 1 provides the proximate analysis of mahua flower sample and from Table no 2, it was observed that TCA – acetone method of protein extraction (method 2) is best amongst the three. The different methods of protein extraction were the *Madhuca ongifolia* flowers are treated with TCA-Acetone and in other method the protein sample were treated with alkaline method. Protein estimation was done by Kjeldhal method in two steps i.e. distillation and titration and then nitrogen content was multiplied with 6.25 to get the final percentage of protein sample. And emulsion activity and emulsion stability was observed for both the treated sample and compared with soy lecithin and bar graph is obtained. It was observed that 0.903(Abs500nm) is highest in 5% and lowest 0.0305(Abs 500nm) in TCA-Acetone activity and stability is maximum at 2% (Abs 500nm) (Table no 5 & 7) and for Alkaline treated sample at 1% it was observed to be 0.450 and at 5% it was highest 0.950 and stability was observed maximum at 4% i.e. 40 (Table no 8 and 9). Emulsion activity was higher in case of 1% i.e. 0.191 and lowest at 5% i.e. 0.180. It was observed that Foaming property of TCA-Acetone treated Mahua flower protein sample to increase from 1% - 5% (Table no 14) and in case of Alkaline treated sample no foaming was observed so a result of which Antifoaming properties is present in that treated sample. Further water holding capacity and Oil holding capacity was observed in both the treated protein sample. The water holding capacity and oil holding a capacity of Mahua after fermented flowers TCA- Acetone treated protein is more than soy lecithin i.e. 2.99 and 2.03. The water holding capacity and oil holding the capacity of Mahua after fermented flowers Alkaline treated protein is more than soy lecithin i.e. 3.97 and 1.032. And lastly water holding capacity in combination of less quality and poor quality of flour is added with both the treated protein sample and it was observed in Alkaline treated protein sample combination has the maximum water holding capacity i.e. 4.544.

Sample	Water holding capacity
TCA-Acetone with flour	4.472
Alkaline method with flour	4.544
Low quality flour	4.28
Poor quality flour	3.767

Table 4: Water holding capacity of Mahua flower for both the treated sample were observed when added in different quality of flour.

5. CONCLUSION

Madhuca longifolia flower sample were crushed into powdered form and proximate analysis was done and it was observed that they have good content of protein. So further work was only done on the protein sample only. Two different treatment was done i.e. TCA-Acetone and alkaline treatment. On comparing the methods, it was found that the TCA-Acetone sample has more protein content and better protein extraction yield than alkaline method and further foaming capacity and foaming stability was observed and later on oil holding capacity and water holding capacity was observed. And lastly protein sample was mixed with poor quality of flour and water holding

capacity was checked and the motive behind carrying out project on mahua is in spite of so many applications still it has failed to make into the commercial uses, only few percentage of local people use it and the local tribes use as a staple food and store this throughout the year to make some earning. It's the government duty to encourage the forest dwellers, local community and tribes to start small scale venture to commercialize the products as pickles, jam, laddu, sauce, juice, biscuits and slowly making into large scale as this will also solve the employment issue in small towns

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School of Biotechnology and Bioinformatics
D Y Patil Deemed to be University,
Navi Mumbai



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**BIOTECHNOLOGY &
BIOINFORMATICS**
NAVI MUMBAI

Plot 50, Sector 15, CBD Belapur,
Navi Mumbai 400614, INDIA
Tel No: +91 22 27567949/48
Email: ncifeh2018@gmail.com