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IN VITRO STUDY AND INSILICO DOCKING OF TURMERIC EXTRACT IN CONTROLLING THE CERVICAL CANCER

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ABSTRACT: Cervical cancer may not cause any symptoms at first, but later, you may have pelvic pain or bleeding. Cell viability was assessed by MTT assay, cell migration was determined by *in vitro* scratch assay and colony formation was determined by clonogenic assay. Tumeric extract and Doxorubicin treatment showed inhibition of cell proliferation in a dose dependent manner. However, combination treatment was found to be more effective in inhibiting cell growth, proliferation and cell migration demonstrating the synergistic effect. To decrease its adverse effects, we performed docking studies with different compounds like curcumin, gallic acid and Doxorubicin with Open Eye software. Homology modeling of Stromelycin has been performed based on the crystal structure of the 1HV5 by using Modeller software. With the aid of the molecular mechanics and molecular dynamics methods, the final model is obtained and is further assessed by procheck and verify 3D graph programs, which showed that the final refined model is reliable. With this model, a flexible docking study of Stromelycin with a group of Gallic acid, Doxorubicin and Curcumin were selected from the previous publications was performed.

KEYWORDS: HeLa, MTT assay, *In vitro* assay, clonogenic assay, Modeling, Docking.

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1.INTRODUCTION

Current cancer treatment strategies favour combination therapies which would offer low toxicities to the cancer patients [1, 2]. Natural products have proven to be the most reliable source of new and effective anticancer agents [3, 4]. Chemotherapy, one type of cancer treatment a major role in

treating various cancers especially to control advanced stages of malignancies in clinical situations [5]. Most of these chemotherapeutic agents show severe normal toxicity, and cause adverse side effects. The clinical use of anthracyclines like doxorubicin induces life threatening cardiomyopathy and congestive heart failure, which is a major difficulty for optimum use of doxorubicin [6]. Tumeric extract inhibits MAPK, p38, c-JNK activation and scavenges reactive oxygen species (ROS) which minimizes the cardiotoxicity of doxorubicin [7] and is a potent suppressor of NFkB pathway which promotes cell proliferation and inhibits apoptosis [8]. GA has been shown to sensitize a variety of human cancer cell lines for apoptosis induced by different anti-cancer drugs [9]. Cancer development involves many aspects of the cell, treatment with a single agent is rarely effective [10]. Two or more cellular processes are usually targeted in therapy. Therefore, combination therapy is now a prominent approach in cancer chemotherapy. Many advantages of this approach There are many advantages of this approach including targeting more than one critical molecular process, delivering lower dose agents with lower toxicity and increasing patient tolerance. Currently there is growing interest in the use of combination chemotherapy allowing the delivery of lower drug dosages each with different modes of activity [11]. Hence, the present study focussed on combinational therapy of tumeric extract, gallic acid and doxorubicin in cervical cancer cells. Tumor resistance to apoptotic cell death is an important hallmark of cancer and contributes to increased survival of cells that have acquired oncogenic mutations, eventually leading to uncontrolled cell proliferation, invasion, metastasis, angiogenesis and chemoresistance.

2. MATERIALS AND METHODS

2.1 Materials

The human cervical cancer cell line (HeLa), Dulbecco's Modified Eagle's medium (DMEM), Fetal bovine serum (FBS) werepurchased from GIBCO Ltd (Life TechnologiesTM., Grand Island, NY). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], Doxorubicin and Gallic acid were purchased from Sigma, St. Louis, MO,USA. sample stock was prepared in dimethyl sulfoxide (DMSO)and doxorubicin and gallic acid were prepared in phosphate buffer saline pH 7.4 (PBS; Gibco) and stored at -20ºC until use. All other chemicals of analytical grade were purchased from Sigma, USA.

2.2 Cell culture and maintenance

HeLa cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, India. They were cultured in Dulbecco's Modified Eagle's mediumwith 10% heat-inactivatedFetal-Bovine Serum, 1.2 g/L NaHCO3 and antibiotic solution (10,000 unitsof Penicillin, 10 mg Streptomycin and 25µg amphotericin / ml). Cells were maintained at37ºC in a humidified incubator containing 95% O2 and 5% CO2. The cells were divided in to six groups as mentioned below and cultured in microplates or T-flasks to conduct different experiments.

Grioup-1: Control(Cntl.) cells

Group-II: Tumeric extract (C) treatedcells

Group-III: Gallic acid (GA) treated cells

Group-IV: Doxorubicin (D) treated cells

Group-V: Tumeric extract (C) + Gallic acid (GA) treated cells

Group-VI: Tumeric extract(C) + Gallic acid (GA) + Doxorubicin (D)treated cells

2.3 Determination of cell viability by MTT assay

Cell viability and cytotoxic effect of tumeric extract, gallic acid and/or doxorubicin on HeLa cells and their proliferation was determined by MTT assay. Based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purpleformazon product [12], cell viability can be assessed. Briefly,5000 cells were seeded ineach well of 96-well plate and treated with desiredconcentrations of tumeric extract (C)(10, 25, 50 and 100μM),doxorubicin (D) (50, 125,250 and 500 nM) and gallic acid (GA) (25, 50, 100, 200μM).Cells were treated with different combinations of C + GA and C + GA +D for 24h and 48 h. Then, 10 μl of MTT (5 mg/ml in PBS) was added to eachwell and incubated for 3-4 h. The medium was removed and 100 μl of 40 mM acid isopropanol was added to each well and after 20 min of mechanicalshaking, the optical density was measured at 570 nm in ELISA platereader.

% Cell Viability = (OD sample)/(OD control) X 100

% Inhibition of proliferation =% untreated cell viability (100) - % drug treated cell viability

2.4 In vitro scratch assay for cell migration

Cells (5×104) were seeded in 60 mm culture plates for monolayer formation up to 80% confluence then scratch was created by sterile p20 tip and washed with 1X PBS to remove floating cells. Cells were treated for 24 h either with tumeric extract (41.28 μM) or doxorubicin (232.34 nM) or gallic acid (105.66 μM)and their combination C + GA (40 + 100μM) and C + GA + D (20 μM + 50μM + 200nM). After incubation the drug containing medium was removed, photographic images were taken at 0, 3, 6, 12 and 24 h using inverted phase microscope. Cell migration was expressed as the percentage of the gap relative to the total area of the cell-free region [13].

2.5 Colony formation assay

Clonogenic assay or colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into colonies. Only a fraction of seeded cells retains the capacity to produce colonies. Before or after treatment, cells are seeded out in appropriate dilutions to form colonies in 5-8 days.Briefly, cells(1000) were seeded in each well of 6 welledculture plates,incubated at 370C with 5% CO2 for 48 h.Then, media was removed,cells were washed with 1X PBS, then treatedwith tumeric extract (41.28 μM) or doxorubicin (232.34 nM) or gallic acid (105.66 μM)and their combinationsas indicated before, for 24 h. Then, media containing compounds/drug was

removed;cells were maintained with fresh media and incubated for 8 days. Colonies were fixed with glutaraldehyde (6% v/v) stained with crystal violet (0.5% w/v) and counted using a stereomicroscope [14].

Calculated plating efficiency (PE) and surviving fraction (SF).

PE = no. of colonies formed/ no. of cells seeded x 100%

SF = no. of colonies formed after treatment/ no. of cells seeded x PE

2.6 3D model building:

The initial model of Stromelysin was built by using homology-modeling methods and the MODELLER software; a program for comparative protein structure modeling optimally satisfying spatial restraints derived from the alignment and expressed as probability density functions (pdfs) for the features restrained. The pdfs restrain Cα-Cα distances, main-chain N-O distances, main-chain and side-chain dihedral angles. The 3D model of a protein is obtained by optimization of the molecular pdf such that the model violates the input restraints as little as possible. The molecular pdf is derived as a combination of pdfs restraining individual spatial features of the whole molecule [15]. The optimization procedure is a variable target function method that applies the conjugate gradients algorithm to positions of all non-hydrogen atoms. The query sequence fromHomo sapiens was submitted to domain fishing server Stromelysin prediction. The predicted domain was searched to find out the related protein structure to be used as a template by the BLAST (Basic Local Alignment Search Tool) program against PDB (Protein Databank). Sequence that showed maximum identity with high score and less e-value were aligned and was used as a reference structure to build a 3D model for Calsium Sensing Receptor. The sequence of Stromelysinwas obtained from NCBI.

The co-ordinates for the structurally conserved regions (SCRs) for Stromelysin were assigned from the template using multiple sequence alignment, based on the Needleman-Wunsch algorithm. The structure having the least modeller objective function, obtained from the modeller was improved by molecular dynamics and equilibration methods using NAMD 2.5 software using CHARMM27 force field for lipids and proteins along with the TIP3Pmodel for water. The energy of the structure was minimized with 1,000,00 steps. A cutoff of 12 Å (switching function starting at 10 Å) for van der Waals interactions was assumed. No periodic boundary conditions were included in this study. An integration time step of 2 fs was used, permitting a multipletime-stepping algorithm to be employed in which interactions involvingcovalent bonds were computed every time step, short-range nonbondedinteractions were computed every two time steps, and long-rangeelectrostatic forces were computed every four time steps. The pair list of the nonbonded interaction was recalculated every ten time steps with a pair list distance of 13.5 Å. The short-range nonbonded interactions were defined as van der Waals and electrostatics interactions between particles within 12 Å. A smoothing function was employed for the van der Waals interactions at a distance of 10 Å. CHARMM27 [force-field parameters were used in all simulations in this study. The equilibrated system was simulated

for 1 ps with a 500 kcal/mol/Å2 restraint on the protein backbone under 1 atm constant pressure and 310 K constant temperature (NPT) and the Langevin damping coefficientwas set to 5 ps unless otherwise stated. Finally, the structure having the least energy with low RMSD (Root Mean Square Deviation) was used for further studies. In this step, the quality of the initial model was improved. The final structure obtained was analyzed by Ramachandran's map using PROCHECK (Programs to check the Stereo chemical Quality of Protein Structures) and environment profile using ERRAT graph (Structure Evaluation server). This model was used for the identification of active site and for docking of the substrate with the enzyme.

2.7 Active site Identification:

Active site of Stromelysin was identified using CASTp server. A new program, CASTp, for automatically locating and measuring protein pockets and cavities, is based on precise computational geometry methods, including alpha shape and discrete flow theory. CASTp identifies and measures pockets and pocket mouth openings, as well as cavities. The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings [16].

2.8 Docking method:

The ligands, including all hydrogen atoms, were built and optimsed with chemsketch software suite. Extremely Fast Rigid Exhaustive Docking (FRED) version 2.1 was used for docking studies (OpenEye Scientific Software, Santa Fe, NM). It is an implementation of multiconformer docking, meaning that a conformational search of the ligand is first carried out, and all relevant low-energy conformations are then rigidly placed in the binding site. This two-step process allows only the remaining six rotational and translational degrees of freedom for the rigid conformer to be considered. The FRED process uses a series of shape-based filters, and the default scoring function is based on Gaussian shape fitting [17].

3. RESULTS AND DISCUSSION

3.1 Assessment of Cell viability and proliferation

Individual combination treatment with tumeric extract, gallic acid and/or doxorubicin enhances the inhibition of HeLa cellproliferation and cell viability, MTT assay was performed [18-21]. Individual treatment with tumeric extract, gallicacid or doxorubicin showed significant inhibition of cellproliferation in a dose dependent manner. Individually, tumeric extract and doxrubicin showed IC50 values at 12.93 µg/mL and 10.45 µg/mL respectively as shown in Table 1.

Table 1: The IC50 of crude extracts of Tumeric extract (µg/mL)

Cell type	Cell line	Tumeric extract	Doxorubicin
Cervical cancer	HeLa	12.93 ± 0.8	10.45± 0.5
Normal cell	L929	33.12 ± 0.82	36.32± 0.7

Table 2 shows the combination treatment with tumeric extract and gallic acid where in tumeric extract (40 μM) and gallic acid (100μM) showed 86.3 % inhibition of cell viability. Interestingly, 91.44 %inhibition of cell viability was noted when tumeric extract (20μM), gallic acid (50μM) and doxorubicin(200nM)was administered indicatingsynergistic effect as shown in Table 2.

Table 2: Effect of curucmin, gallic acid and/or doxorubiin treatment on cell viability of HeLa cells at 24h.

Cell type	Cell line	Curcumin+gallic acid	Doxorubicin
Cervical cancer	HeLa	11.34± 0.8	10.45± 0.5
Normal cell	L929	30.24± 0.82	36.32± 0.7

3.2 Assessment of cell migration

The % of cells thathad migrated into the scratch area wascalculated after 3, 6, 12 and 24 h and shown in figure 3. Complete gap closure in 24 hours was observed in the untreated cells,however, significant changein cell migration was observed when treated with tumeric extract, gallicacid and doxorubicin in the concentrations mentioned before, either individually or in combinations [22, 23]. Control, tumeric extract, doxorubicin and gallic acid individually shown inhibition of cell migration by o.15, 0.75, 0.60 and 0.75 inches respectively. In combination treatment, C + GA shown 0.80 inches inhibition of cell migration while C+GA+ D shown0.85 inches. These results suggest that combinationtreatment was more effective in inhibiting themigration of HeLa cells compared toindividual drug treatments (Fig 3).

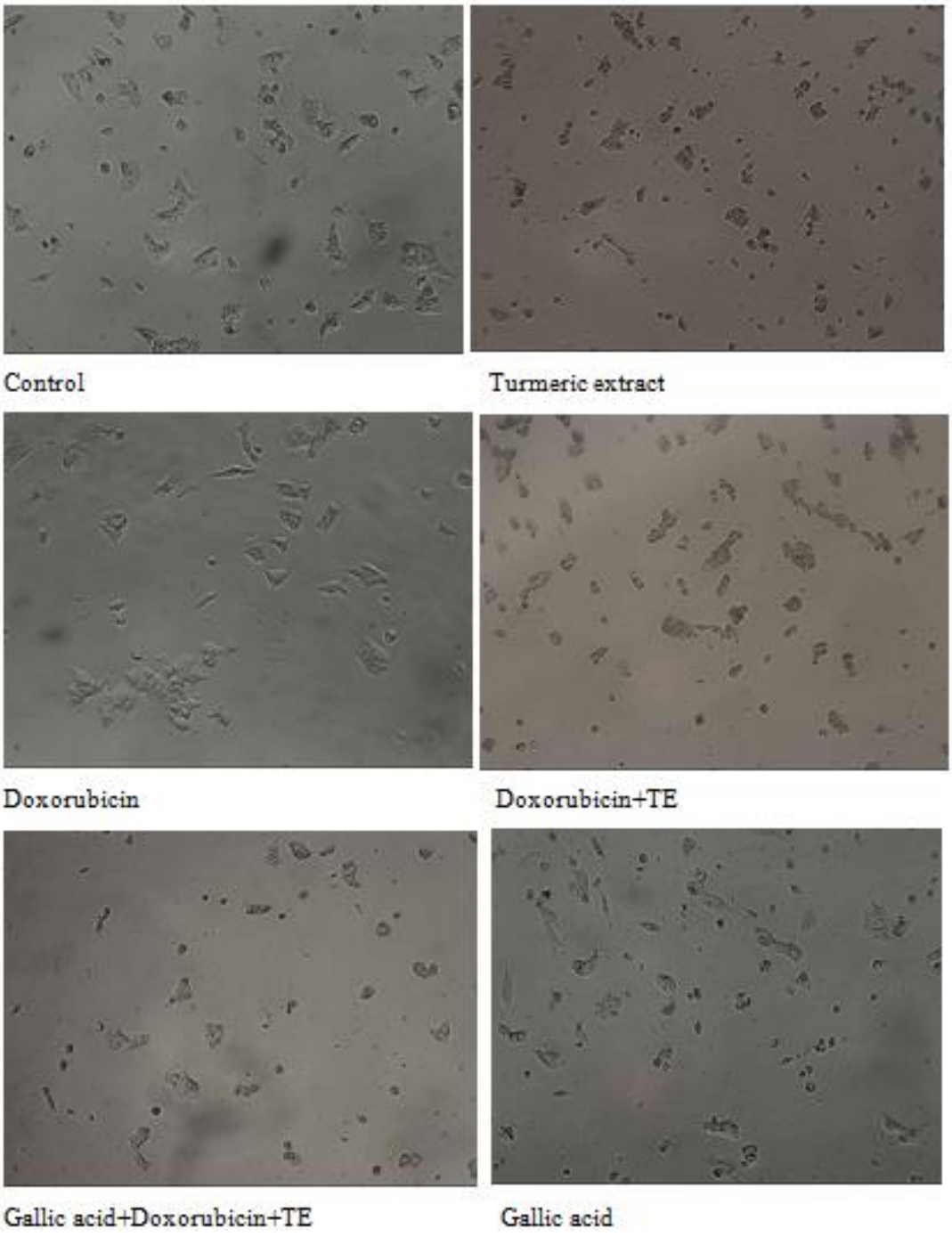

Figure 3: Effect of Turmeric extract, gallic acid and/or doxorubicin on cell migration.

3.3 HeLa cell colonies formation

The effect of tumeric extract, gallic acidand doxorubicin on HeLa cells colony formation was assessed. The % of cells that had formed colonies after 8 days wascalculated and shown in figure 4. There were more colonies observed in untreated control cells whilesignificant changes in colonies formation were observed in cells treated with tumeric extract, gallic acid and doxorubicinin concentrations mentioned before, both individually and in combinations.Tumeric extract, gallic acid and doxorubicin individually shown 66.67%, 61.11%, 75% colony formation, while C+GA shown 47.22% and C+GA+D shown 30.56% colony formation. These results suggest that

combinationtreatment was more efficacious in inhibiting colony formation of HeLa cells indicating the synergistic effect (Fig 4).

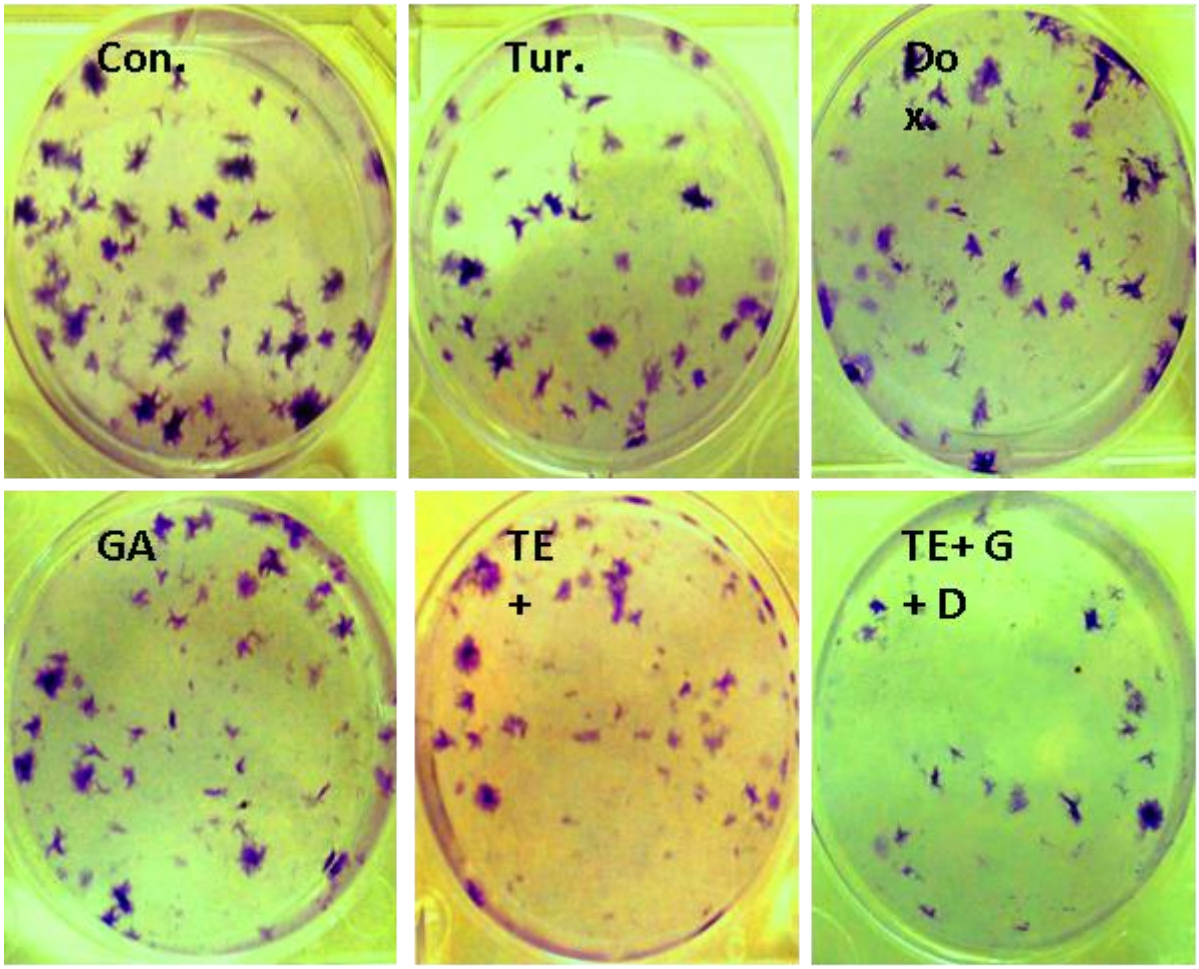

Fig 4: Studies of different combinations on cell colonies growth

3.4 Homology Modeling of Stromelysin:

A high level of sequence identity should guarantee more accurate alignment between the target sequence and template structure. In the results of BLAST search against PDB, only one-reference protein 1HV5 Chain A has a high level of sequence identity and the identity of the reference protein with the Stromelysin protein are 94%. Structurally conserved regions (SCRs) for the model and the template were determined by superimposition of the two structures and multiple sequence alignment [24].

In the following study, we have chosen 1HV5 chain A as a reference structure for modeling Stromelysin domain. Coordinates from the reference protein (1HV5chain A) to the SCRs, structurally variable regions (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints (Fig 5).

> EBI > Tools > Multiple Sequence Alignment > ClustalW2

ClustalW2 Results

Alignments | Result Summary | Guide Tree | Submission Details | Submit Another Job

Alignment

View Alignment File | Hide Colors

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CLUSTAL 2.1 multiple sequence alignment

domain          ----SGGRWEKTDLTYRILRFPWQLVQEQVRQTMAEALKVWSDVTPLTFTEVHEGRADIM 56
1HV5            MFVLSGGRWEKTDLTYRILRFPWQLVREQVRQTVAEALQVWSEVTPLTFTEVHEGRADIM 60
                    ***********************:******:****:***:******************

domain          IDFARYWHGDDLPFDGPGGILAHAFFPKTHREGDVHFDYDETWTIGDDQGTDLLQVAAHE 116
1HV5            IDFARYWHGDNLPFDGPGGILAHAFFPKTHREGDVHFDYDETWTIGDNQGTDLLQVAAHE 120
                **********:**************************************:************

domain          FGHVLGLQHTTAAKALMSAFYTFRYPLSLSPDDCRGVQHLYGQPW 161
1HV5            FGHVLGLQHTTAAKALMSPFYTFRYPLSLSPDDRRGIQHLYG--- 162
                ******************.************** **:******

PLEASE NOTE: Showing colors on large alignments is slow.
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Fig 5: alignment of Stromelysin with template 1HV5 chain A.

In the modeler we will get a 20 PDB out of which we select a least energy .The energy unit will be in kilo joule .All side chains of the model protein were set by rotamers [25]. The final stable structure of the Stromelysin obtained is shown in Figure 6. By the help of SPDBV it is evident that Stromelysin protein has 03 helices and 7 sheets and it is shown in the Figure 7.

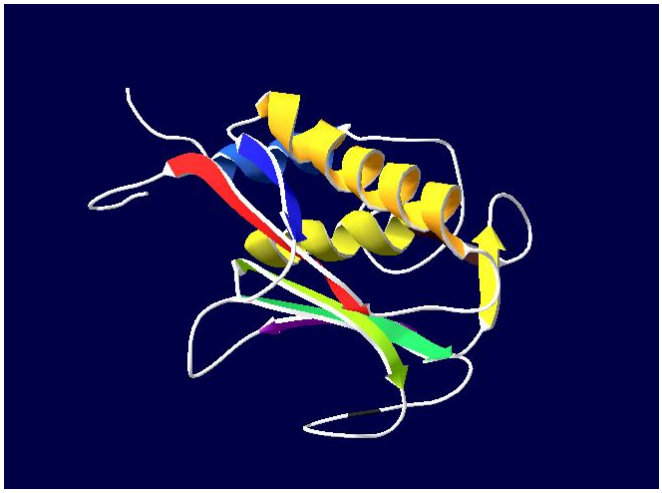

Fig 6: Protein with helices and sheets

The structure having the least energy with low RMSD (Root Mean Square Deviation) which was obtained by the NAMD is in water molecule (TIP3).

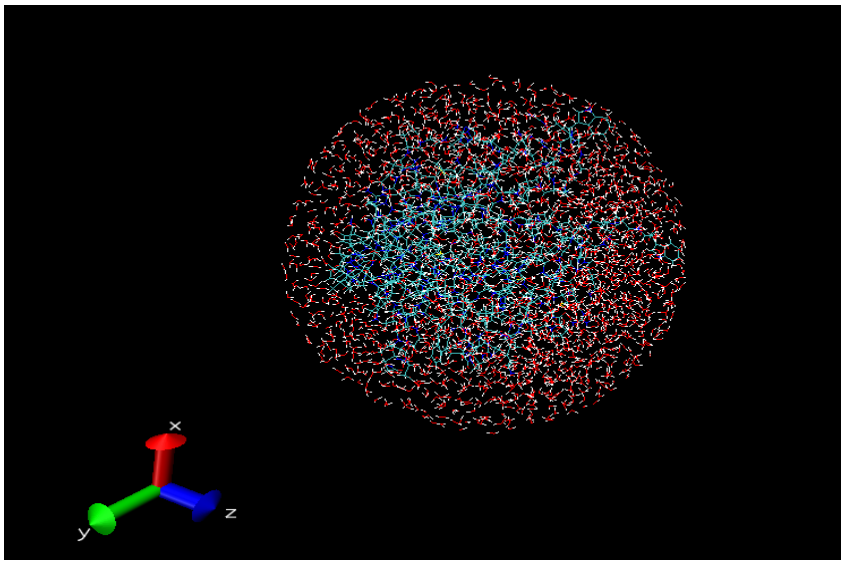

Fig 7: Stromelysin with water molecule.

The final structure was further checked by verify 3D graph and the results have been shown in Figure 8. The overall scores indicates acceptable protein environment.

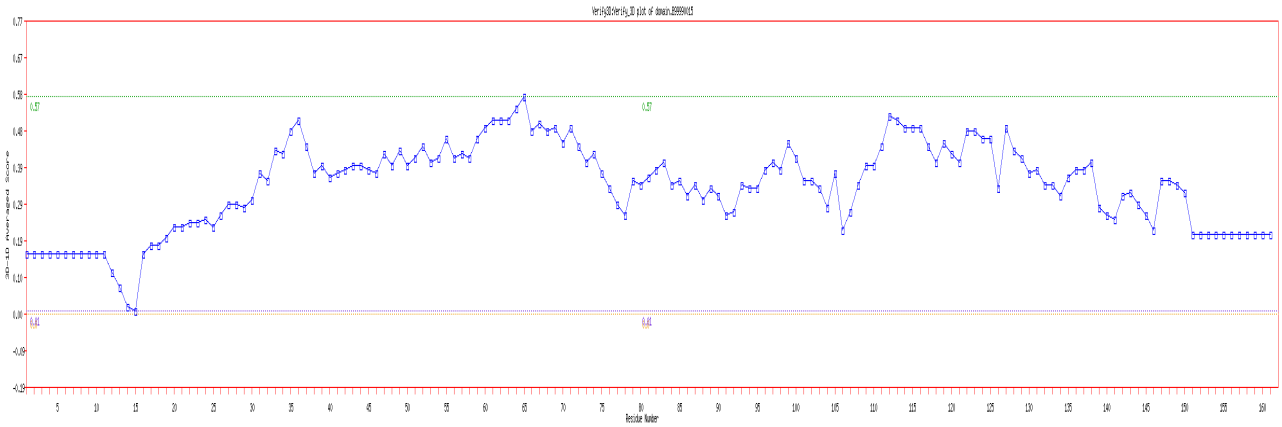

Figure 8: The 3D profiles verified results of Stromelysin model; overall quality score indicates residues are reasonably folded.

3.5 Validation of Protien:

After the refinement process, validation of the model was carried outusing Ramachandran plot calculations computed with the PROCHECKprogram. The π and ψ distributions of the Ramachandranplots of non-glycine, non-proline residues are summarized [26-29]. Altogether 93% of the residues of Stromelysin was infavored and allowed regions. The overall PROCHECK G-factor of Stromelysin nd verify3D environment profile were good (Fig 9).

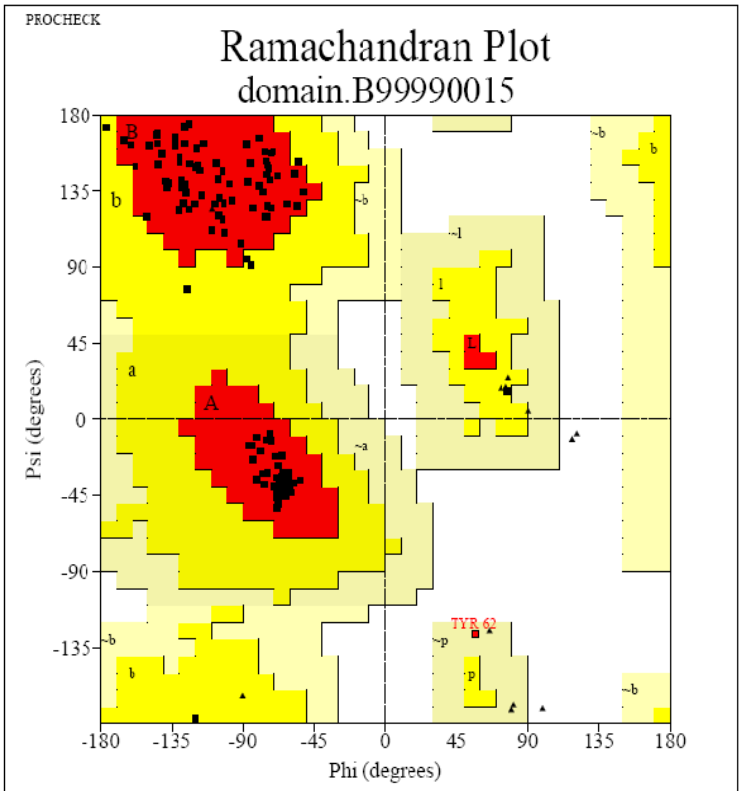

Figure 9: Ramachandran Plot

3.6 Superimposition of 1HV5 chain A with Stromelysin Protein:

The structural superimposition of C trace of template and Stromelysin is shown in Figure 10. The weighted root mean square deviation of C α trace between the template and final refined models 1.29A°. This final refined model was used for the identification of active site and for docking of the substrate with the protein *Stromelysin (Fig 10).*

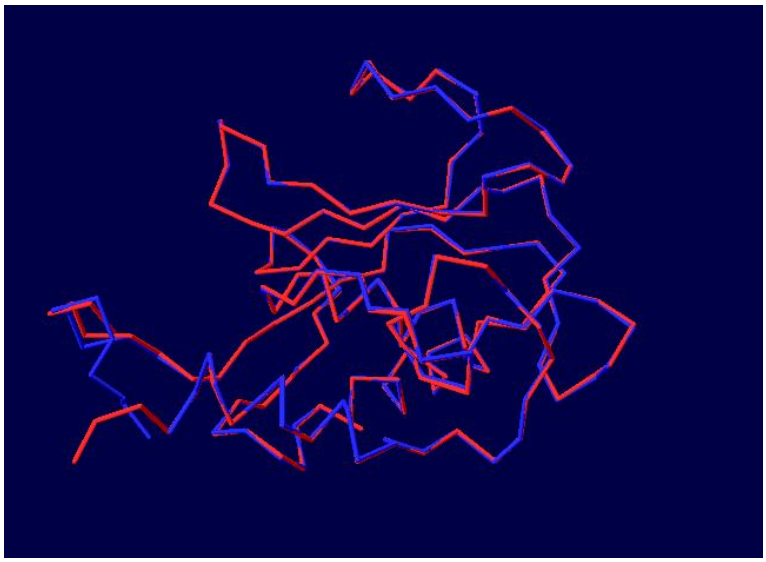

Fig 10: Super imposition of Stromeylysin(Red) and 1HV5(Blue) chain A.

3.7 Active site Identification of Stromelysin protein:

After the final model was built, the possible binding sites of Stromelysin was searched based on the

structural comparison of template and the model build and also with CASTp server and was shown in Figure 11. Since, Stromelysin and the 1HV5 chain A are well conserved in both sequence and structure; their biological function should be identical [30-33]. Infact from the structure-structure comparison of template, final refined model of Stromelysin protein using SPDBV program and was shown in Figure9. It was found that secondary structures are highly conserved and the residues GLY75, ILE76, LEU77, ALA78, HIS79, GLN111, VAL112, HIS115, GLU116, HIS119, HIS125, LYS130, ALA131, LEU132, SER134, ALA135, PHE136, TYR137, THR138, ARG140, PRO142, LEU143, SER144, LEU145, SER146.

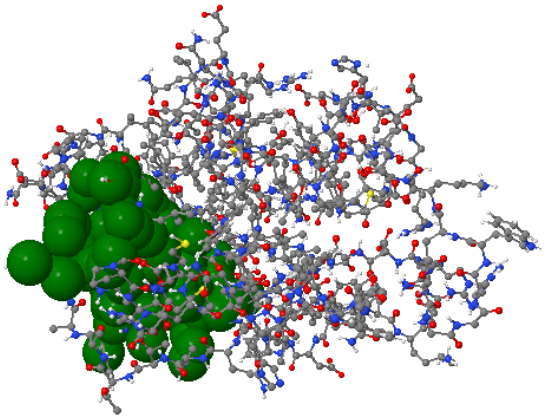

Figure 11: active site of Stromelysin.

The Ligand (inhibitor) molecules used for Docking studies (Fig 12a-c)

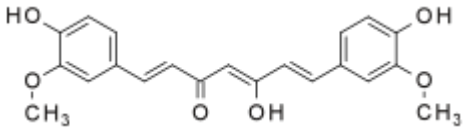

Fig 12a : Structure of Curcumin

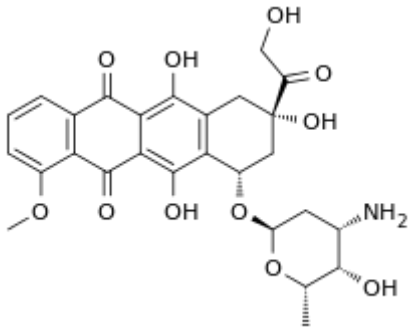

Fig 12b: Structure of Doxorubicin

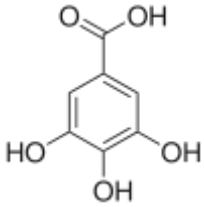

Fig 12c: Structure of Gallic acid

3.8 Docking of inhibitors with the active site of Stromelysin:

Docking of the inhibitors with protein Stromelysin was performed using FRED v 2.1, which is based on Rigid Body Shape-Fitting (Open Eye Scientific Software, Santa Fe, NM). This program generates an ensemble of different rigid body orientations (poses) for each compound conformer within the binding pocket and then passes each molecule against a negative image of the binding site [34, 35]. Poses clashing with this 'bump map' are eliminated. Poses surviving the bump test are then scored and ranked with a Gaussian shape function. We defined the binding pocket using the ligand-free protein structure and a box enclosing the binding site. This box was defined by extending the size of a cocrystalized ligand by 4 Å (addbox parameter of FRED). This dimension was considered here appropriate to allow, for instance, compounds larger than the cocrystallized ones to fit into the binding site. One unique pose for each of the best-scored compounds was saved for the subsequent steps. The compounds used for docking was converted in 3D with OMEGA (same protocol as above)(OpenEye Scientific Software, Santa Fe, NM). To this set, the substrate (generation of multiconformer with Omega) corresponding to the modeled protein were added (Fig 13a-c and table 3).

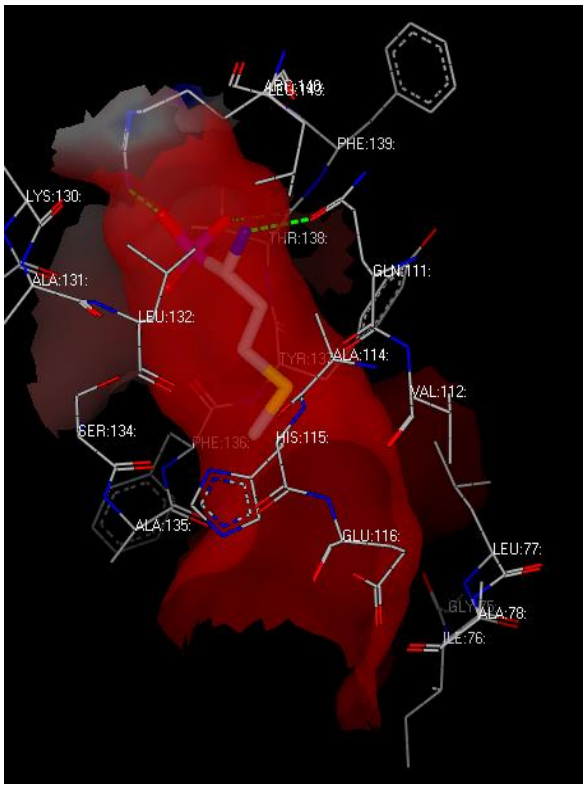

Fig 13a: Docking of Curcumin

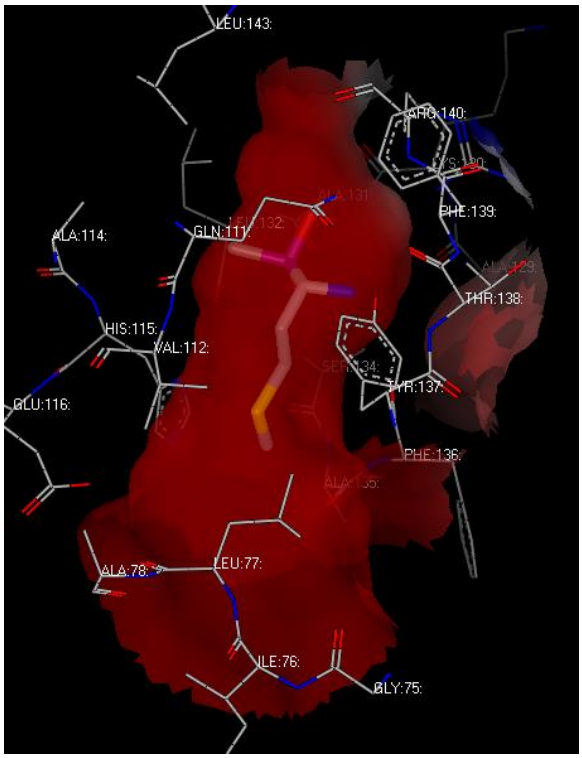

Fig 13b: Docking of Doxorubicin

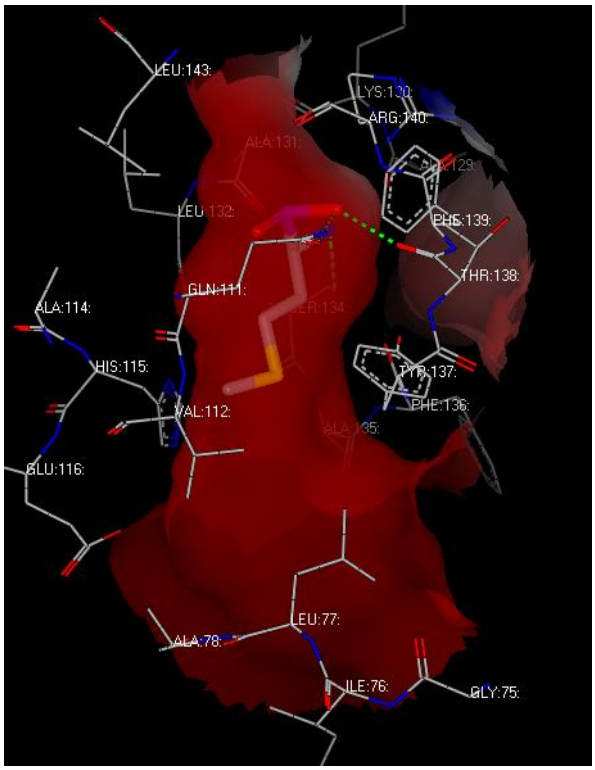

Fig 13c: Docking of Gallic acid

Table 3: Docking scores of the compounds with target protein

Total Score (KJ/mol)	Molecule
-304.05	Curcumin
-240.59	Doxorubicin
-239.59	Gallic acid

4. CONCLUSION

In the present study, turmeric extract, GA and exhibited significant suppressive effect on HeLa cellsviability in a dose dependent manner. Suchan inhibitory activity of cell viability was enhanced significantly when the HeLa cells wereexposed to C + GA and C + GA+ D combinations. Cell migration was significantly inhibited 60% with C + GA and 65% with C + GA + D combination.Results of the presentstudy revealed that combination exposure of both drugsat same time point exhibited highest inhibition in cellviability, colony formation and cell migration than either C, GA or D alone.These observations may be of value while carefully considering the combination therapies in a clinical settingand beneficial in cervical cancer therapy. Our results suggest that concurrent treatment of C, GA and D shown synergistic effect in human cervical cancer (HeLa) cells and these drugs may havefuture clinical utility for treating cervical cancer.

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CONFLICT OF INTEREST

The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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