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Research Article

IN-SILICO DRUG DESIGNING USING B-SITOSTEROL ISOLATED FROM *FLAVERIA TRINERVIA* AGAINST PEPTIDE DEFORMYLASE PROTEIN TO HYPOTHESIZE BACTERICIDAL EFFECT

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ABSTRACT

β-sitosterol, pearl white compound was isolated from the chloroform extract of *Flaveria trinervia* (Asteraceae). The isolated compound was characterized by IR, ^{1}H-NMR, and mass spectral studies. The antibacterial activity was screened against 20 clinical strains belonging to *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi, Salmonella paratyphi, Echerichia coli,* and *Staphylococcus aureus* isolated from different infectious sources. Minimum inhibitory concentration (MIC) was carried out against clinical isolates using two fold agar dilution method. β-sitosterol exhibited significant zone of inhibition against all the clinical isolates except *S. typhi* and *S. paratyphi*. *In-silico* drug designing using β-sitosterol for antibacterial activity was investigated by docking β-sitosterol molecule with the peptide deformylase protein (PDF) and compared with the commercial antibiotic ciprofloxacin. The active domain of PDF consisted of Gly45(A), Gln50(A), Leu91(A), Glu133(A) as catalytic residues with residues Gly43(A), Gln42(A), Arg97(A), Glu88(A), His132(A), Cys129(A), Glu133(A), Gln50(A), Leu91(A), His136(A), Cys90(A), Ile44(A), Gly89(A) in its active pocket, This pocket was docked with β-sitosterol and ciprofloxacin at the torsional degree of freedom 0.5 units with genetic algorithm resulted with the inhibition constant as $2.4x10^{-7}$ for β-sitosterol. Where as, inhibition constant of ciprofloxacin was $2.73x10^{-8}$.

Keywords*:* β-sitosterol, *In-Silico* docking, *Flaveria trinervia*, Antibacterial activity, Peptide deformylase, Chloroform extract.

INTRODUCTION

New antibiotics that are active against resistant bacteria are required to combat with the present scenario of bacterial pathogenesis. Bacteria have lived on earth since several billion years. During this time, they encountered a wide range of naturally occurring antibiotics. To survive bacteria developed antibiotic resistance mechanism [1]. Many reports are available on the uses of medicinal plants against pathogenic microorganisms with multiple resistances to third and fourth generation antibiotics [2,3].

Flaveria trinervia spring C. Mohr (Asterecae) population that grow only in alkaline soil [pH 7.2-8.2], mainly in marshy land near Chitradurga Dist, Karnataka State, India. This plant is locally referred as Bellary halabu or katthe kivi gida. Traditionally it is used as a promising drug as antimicrobial agent for infectious wounds in Karnataka state, India. To justify the ethnomedical claims, chloroform extract and the isolated phytoconstituent β-sitosterol of *F. trinervia* were screened for antibacterial activity.

Apart from existing therapies literature survey reveals that antibacterial therapy could be included for treating bacterial infections and many of the antibacterial agents, including those obtained from the herbal source are being used successfully for treating bacterial infections. Hence, the present investigation was carried out to evaluate the antibacterial activity of chloroform extract of *Flaveria trinervia* and the isolated phytoconstituent β-sitosterol against 20 clinical strains belonging to *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Salmonella typhi, Salmonella paratyphi, Echerichia coli,* and *Staphylococcus aureus* isolated from different infectious sources.

Peptide deformylase (PDF; EC 3.5.1.31) fulfills the antibacterial criterion, as it is likely the most attractive bacterial target to deliver the next class of novel antibacterial drugs [4]. Over three decades ago, it was realized that bacterial protein synthesis starts with a N-formylated methionine but finally yields proteins mostly lacking this N-acylated initiator [5-7]. PDF is present in sequenced bacterial genomes and deformylation of newly synthesized peptides is essential in bacterial growth [5, 8-10].

In the present investigation, the antibacterial activity of chloroform extract and β-sitosterol was evaluated and the mode of action of the β-sitosterol molecule was hypothesized by *in-silico* docking of β-sitosterol molecule to peptide deformylase protein, an important enzyme whose inhibition promotes inhibitions of bacterial cell growth. This is because the *N*-formyl methionine of the nascent protein in bacteria is removed by the sequential action of PDF in order to convert the nascent protein to mature protein. This formylation-deformylation cycle is essential for bacterial growth. Hence, the minimum inhibitory concentration (MIC) evaluation and molecular docking studies were also carried out to support this investigation.

MATERIALS AND METHODS

Drugs and chemicals

The standard drug ciprofloxacin (Usan pharmaceuticals Pvt, Ltd. Maharastra, India) was used for evaluation of bactericidal activity. Chloroform, Hexane, ethyl acetate and DMSO (Merck, India).

Plant resource

F. trinervia herb was collected from the agricultural fields near Chitradurga city of Karnataka, India. Plant was authenticated by Dr. Manjunath by comparing with the voucher specimen deposited at Kuvempu University herbarium specimen FDD-No. 53 [11].

Isolation and phytochemical investigation

Fresh whole plant material was shade dried, powdered mechanically and was subjected for soxhlet extraction using chloroform as solvent system for about 48 h. The extract was filtered and concentrated in vaccum under reduced pressure using rotary flash evaporator (Buchi, Flawil, Switzerland) and allowed it for complete evaporation of the solvent. Chloroform extract was vacuum dried.

Crude chloroform extract of whole plant was studied for its phytoconstituent profile that could be separated using thin layer chromatography (TLC) studies, which showed separation of a single compound as a single spot with the R_f value 0.42 using the solvent system hexane : ethyl acetate in the ratio 9:1. Using the same solvent proportion the active constituent was separated by column chromatography (180 g silica gel of 60 -120 mesh, 60x4 cm), fractions were collected at the interval of 5 ml. Meanwhile, the fractions were monitored by TLC to check the purity of separated constituent, which confirmed the single spot. The isolated compound gave red colour in salkowski's test and green colour in Liebermann-Burchard test indicated the compound was sterol and the melting point (m.p.) was observed to be 130-135^{o}C. Characterization of the

isolated compound was carried by IR, ^{1}HNMR and mass spectral studies.

Determination of minimum inhibitory concentrations (MIC)

The agar dilution susceptibility test was performed based on modified method of NCCLS, 2003 [12] and CLSI, 2009 [13] to determine the MIC. The chloroform extract and β-sitosterol were dissolved in sterilized 5% dimethyl sulfoxide (DMSO; that enhances compound solubility) (800 mg/ml concentration) and were taken as standard stock. A series of two fold dilutions of each extract with a final concentration of 80, 40, 20, 10 and 5 mg/ml were prepared in nutrient agar. After solidification, the plates were spotted with 100 µl of overnight grown bacterial cultures approximately containing 1 × 10^4 CFU/ml. The test was carried out in triplicates. The plates were incubated overnight at 37°C. After 18 – 24 h, the MIC was determined.

Antibacterial screening

The antibacterial activity of the chloroform extract and β-sitosterol was screened by agar well radial diffusion method against twenty clinical isolates of bacterial strains belonging to *Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi, Salmonella paratyphi* and *Escherchia coli* respectively [14]. The bacterial strains were collected from different infectious status of the patients with the help of authorized physicians, in district health center of Gulburga, Karnataka state, India. The clinical isolates were identified in Microbiology Laboratory, Gulburga University following the standard method [15]. The bacterial suspensions were diluted in 10^{-1} to 10^{-8} phosphate buffered saline. Samples were homogenized and then loaded in six aliquots of 20 µl each onto nutrient agar plates. The working cultures were prepared by inoculating a loopful of each test microorganism in 3 ml of nutrient broth (NB) from NA slants. Broths were incubated at 37°C for 24 h. The suspension was diluted with sterile distilled water to obtain approximately 10^6 CFU/ml.

The chloroform extract and β-sitosterol were dissolved in 5% aqueous DMSO to get stock solutions. Commercial bactericide ciprofloxacin was used as standard (100 µg/100 µl of sterilized distilled water) concomitantly with the test samples. The activity was screened comparatively with the reference ATCC strains (*Pseudomonas aeruginosa*- ATCC-20852; *Staphylococcus aureus*- ATCC 29737), (*Salmonella typhi* – ATCC-19430), (*Salmonella paratyphi* – ATCC-9150), (*E. coli* – ATCC-25922) and MTCC strains (*Klebsiella pneumoniae* – MTCC-618).

Sensitive agar well radial diffusion technique was used for the assessment of antibacterial activity of the test samples. Sterilized nutrient agar medium was poured into sterilized petridishes. Nutrient broth containing 100 µl of 24 h incubated cultures of clinical isolates was spread on the agar medium. Wells were created using a sterilized cork borer in an aseptic condition. 100 µl of crude chloroform extract, 100 µl of β-sitosterol and 100 µl of standard drug ciprofloxacin were loaded on to their corresponding wells. The plates were incubated at 37°C for 24 h. The diameter of the zone of complete inhibition of the bacteria was measured around each well and readings were recorded in mm. The results of these experiments were expressed as mean ± S.E.M. of three replicates in each test [16].

Statistical Analysis

The data of bactericidal evaluations were expressed as mean ± S.E.M of three replicates. The statistical analysis was carried out using one way ANOVA followed by Tukey's *t*-test. The difference in values at $P< 0.01$ was considered as statistically significant. The analysis of variance (ANOVA) was performed using ezANOVA (version 0.98) software to determine the mean and standard errors of the experiments.

***In-silico* drug designing by molecular docking**

Automated computational docking was used to determine the orientation of inhibitors bound in the active site of PDF protein. A lamarckian genetic algorithm method, implemented in the program AutoDock 3.0, was employed [17]. The ligand molecules, β-sitosterol and ciprofloxacin were designed and the structure was analyzed by using ChemDraw Ultra 6.0. 3D coordinates were prepared using PRODRG server [18]. The protein structure file 1G27 was taken from PDB (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C terminal oxygen [19]. For docking calculations, Gasteigere Marsili partial charges were assigned to the ligands and nonpolar hydrogen atoms were merged [20]. All torsions were allowed to rotate during docking. The grid map, which was centered at the following active pocket residues of the protein PDF (Gly43(A), Gln42(A), Arg97(A), Glu88(A), His132(A), Cys129(A), Glu133(A), Gly45(A), Gln50(A), Leu91(A), His136(A), Cys90(A), Ile44(A), Gly89(A)) were predicted from the CASTp server were generated with AutoGrid. The Lamarckian genetic algorithm was applied for minimization, using default parameters. The number of docking runs was 10. Number of energy evaluations was 100,000 and the maximum number of iterations 10,000.

RESULTS AND DISCUSSION

Isolation and phytochemical investigation

The pearl white compound eluted from the chloroform extract of *Flaveria trinervia* was tested for qualitative chemical analysis showed positive for sterols. The isolated constituent was characterized by IR, ^{1}H NMR and Mass spectral analysis. In IR a spectrum absorption frequency was observed at 3445.18 cm^{-1} (br, OH), 2928.21 cm^{-1}, 2254.99 cm^{-1}(C-H str. in CH_3 and CH_2), 2131.53 cm^{-1} (C-H str. in CH_2), 1651.22 cm^{-1}, 1005.00 cm^{-1} (C-O str. of secondary alcohol). The ^{1}H NMR spectrum showed signals at δ 0.64 to 1.006 (18 H,6 X CH_3) δ1.029 to 1.23 (22 H,11XCH_2) δ 1.44 to 2.28 (m, 8H, methane protons) δ 3.5 (m, 1H, OH), δ5.4 (m, 1H, vinylic proton). Mass Spectrum showed the peak at *m/z* 414 indicating the molecular weight of the compound. Melting point of this compound was about 130-135°C. Based on the above data the compound was characterized as β-sitosterol.

Evaluation of minimum inhibitory concentrations (MIC)

The MIC values of both chloroform extract and β-sitosterol ranged from 5 to 80 mg/ml (Table 1). Chloroform extract of *F. trinervia* showed significant inhibition at MIC 05 mg/ml against *E. coli* and *Staphylococcus aureus.* Whereas, for *K. pneumonia* and *P. aureginosa* inhibition was observed at 20 mg/ml. β-sitosterol showed maximum activity against *Staphylococcus aureus* at MIC 05 mg/ml. Highest MIC of β-sitosterol was observed against *Salmonella typhi* and *Salmonella paratyphi* at 80 mg/ml.

Table 1: *In vitro* minimum inhibition concentrations evaluation of chloroform extract and β-sitosterol of *Flaveria trinveria* against *Staphylococcus aureus, Pseudomonas aureginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella paratyphi* and *Escherchia coli*

Test pathogenic bacteria	Strain	*Flaveria trinervia* plant	
		Chloroform extract	β-sitosterol
Staphylococcus aureus	ATCC-29737	05	05
Pseudomonas aureginosa	ATCC-20852	20	10
Klebsiella pneumonia	MTCC-618	20	40
Salmonella typhi	ATCC-19430	80	80
Salmonella paratyphi	ATCC-9150	*	80
Escherchia coli	ATCC-25922	05	20

** indicates values more than 80 mg/ml. The value of each constituents consisted of* mean *± S.E.M. of 03 replicates. ND – Not Defined.*

Table 2: In vitro antibacterial activity of chloroform extract and its constituent β-sitosterol of Flaveria trinveria against Staphylococcus aureus, Pseudomonas aureginosa, Klebsiella pneumoniae, Salmonella typhi, Salmonella typhi, Salmonella paratyphi and Escherchia coli

Pathogens	Bacterial strains tested	Source of collection	Chloroform extract	β-sitosterol	Reference drug Ciprofloxacin
	Zone of inhibition (in mm)				
Staphylococcus aureus					
	Sa-1	ATCC-29737	19 ± 0.58	17.83 ± 0.58	21±0.86
	Sa-2	Abscess	18.67 ±0.33	18.33 ± 0.88	21±1.63
	Sa-3	Urine	21.83 ± 1.3	ND	19.5±0.96
	Sa-4	Wound	18.17 ± 0.84	19.17 ± 1.33	18.67±1.41
	Sa-5	Hospital effluents	20.33 ± 1.76	15.5 ± 2.84	21±1.81
Pseudomonas aureginosa					
	Pa-1	ATCC-20852	13.33 ± 0.88	16.11 ± 1.41	22.67±0.8
	Pa-2	Urine	16 ± 0.58	ND	21±1.13
	Pa-3	Pus	14.33 ± 1.2	15.33 ± 0.58	18.83±1.01
	Pa-4	Stool	15.17 ± 0.33	16.67 ± 0.33	19.17±0.95
Klebsiella pneumoniae					
	Kp-1	MTCC-618	15.33 ± 1.45	12 ± 1.86	16±0.58
	Kp-2	Urine	ND	11.33 ± 0.88	16.83±1.4
	Kp-3	Feaces	14.5 ± 0.84	ND	14.33±1.31
	Kp-4	Sputum	16.83 ± 1.3	11.67 ± 1.76	16±1.32
Salmonella typhi					
	St-1	ATCC-19430	6.17 ± 1.45	5.33 ± 1.2	21±0.58
	St-2	Blood clot	9 ± 1.96	4.17 ± 0.33	20.83±0.98
Salmonella paratyphi					
	Spt-1	ATCC-9150	4.83 ± 1.31	6 ± 0.33	16.83±1.3
	Spt-2	Blood clot	ND	ND	20±1.06
Escherchia coli					
	Ec-1	ATCC-25922	19 ± 0.58	14.5 ± 1.84	17.33±0.49
	Ec-2	Hospital effluents	20 ±0.58	15.17 ± 1.2	16.33±1.76
	Ec-3	Urine	17.67 ± 1.45	16.83 ± 0.33	20±1.06

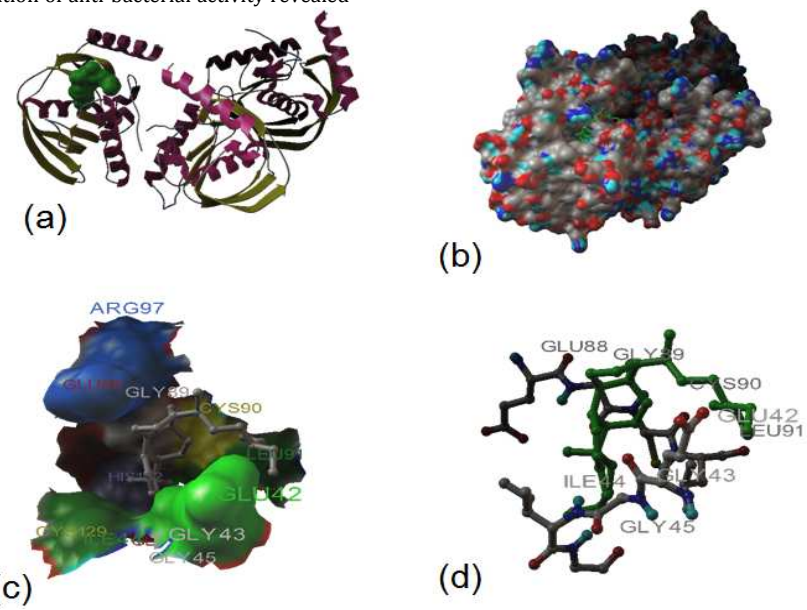
The value of each constituents consisted of ± S.E.M. of 03 replicates. ND – Not Defined.

Antibacterial screening

Chloroform extract showed significant results in inhibiting *S. aureus* with (19 ± 0.58) mm zone of inhibition when compared to the β-sitosterol (17.83 ± 0.58 mm). β-sitosterol against *P. aureginosa* produced 16.11 ± 1.41 mm zone of inhibition whereas, chloroform extract showed 13.33 ± 0.88 mm.

Chloroform extract was potent against *K. pneumoniae* (15.33 ± 1.45 mm) than β-sitosterol (12 ± 1.86 mm). β-sitosterol proved to be less significant against clinical isolates of *S. typhi* and *S. paratyphi*. Chloroform extract also showed better inhibition of *S. typhi* (6.17 ± 1.45 mm) and *E. coli* (19 ± 0.58 mm). β-sitosterol inhibited the growth of *E. coli* (14.5 ± 1.84 mm), this inhibition was less efficient the chloroform extract. Evaluation of anti-bacterial activity revealed that the chloroform extract and β-sitosterol showed effective activity against all the six bacterial pathogens. Specifically, chloroform extract was more efficient than β-sitosterol but less potent than standard drug ciprofloxacin. This significant effect of the phytoextract is due to the presence of a single active constituent in higher levels or due to the combined effect of more than one phytoconstituent. Results of *in-vitro* antibacterial activity are depicted in Table 2.

Both chloroform extract and β-sitosterol were significantly effective in controlling the growth of all the bacterial strains under study. This investigation supported the ethnomedical claims of *F. trinveria* as a potent antibacterial plant. This is mainly due to the presence of β-sitosterol, which proved to be potent against bacteria.



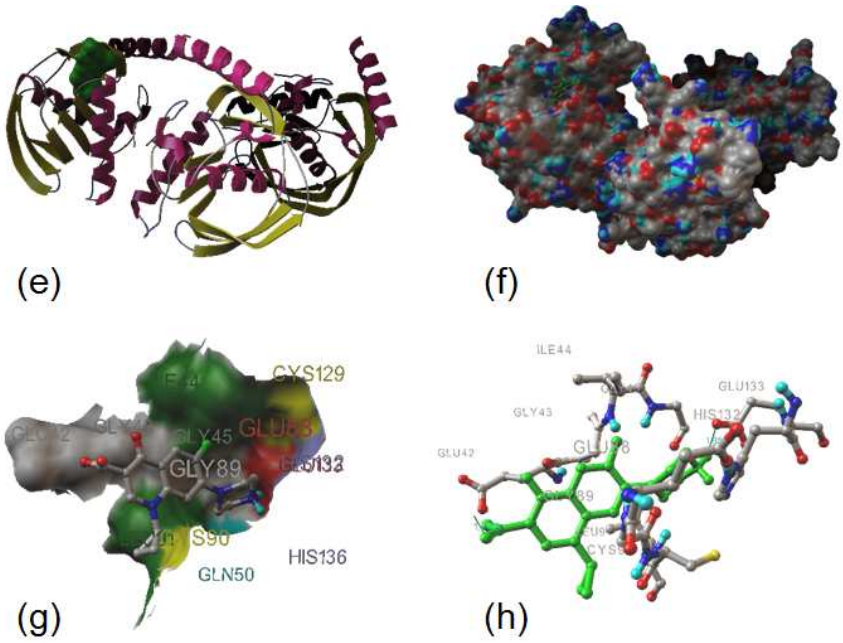


Fig. 1: ***(a) Ribbon model of GSK3-b showing the interaction of β-sitosterol (MSMS-MOL) with the amino acids in the binding pocket predicted by CASTp server. (b) Molecular surface model of PDF with the bound β-sitosterol (blue ball and stick). (c) The β-sitosterol molecule enfolded in the active pocket of PDF showing interacting amino acids: Gly43(A), Gln42(A), Arg97(A), Glu88(A), His132(A), Cys129(A), Glu133(A), Gly45(A), Gln50(A), Leu91(A), His136(A), Cys90(A), Ile44(A), Gly89(A) (MSMS-MOL). (d) Stick and ball model of β-sitosterol molecule (green) hydrogen bonded with the PDF with Glu 133 and showing the residues in the linker/hinge region and those involved in the important ion pair interaction (Glu 42, Gly 43, Ile 44, Gly 45, Glu 88, Gly 89, Cys 90 and Leu 91) are shown. (e) Ribbon model of PDF showing the interaction of ciprofloxacin (MSMS-MOL) with the amino acids in the binding pocket predicted by CASTp server. (f) Molecular surface model of PDF with the bound ciprofloxacin (blue ball and stick). (g) The molecule ciprofloxacin enfolded in the active pocket of PDF showing interacting amino acids: Gly43(A), Gln42(A), Arg97(A), Glu88(A), His132(A), Cys129(A), Glu133(A), Gly45(A), Gln50(A), Leu91(A), His136(A), Cys90(A), Ile44(A), Gly89(A) (MSMS-MOL). (h) Hydrogen bond between the ciprofloxacin (stick and ball coloured by green) and the Glu 133 residue of PDF.***

In-Silico drug designing by molecular docking

Comparative computational docking of β-sitosterol and standard drug ciprofloxacin to the peptide deformylase protein revealed that the docked energy for the β-sitosterol was -10.55 with an estimated inhibition constant of 2.4 x 10^{-7} and intermolecular energy -10.9 (Figure 1a). The docked energy of ciprofloxacin was only -11.14 with an inhibition constant of 2.73 x 10^{-8} and intermolecular energy of 0.11. β-sitosterol was completely enfolded in the entire active pocket of PDF (Figure 1b) as compared to ciprofloxacin. The β-sitosterol (ball and stick indicated grey) found to sit in the proper orientation complementary to the topology of the site (residues shown in MSMS-MOL) (Figure 1c). However, the orientation of β-sitosterol molecule was perpendicular to the plane made by Glu 42, Gly 43, Ile 44, Gly 45, Glu 88, Gly 89, Cys 90 and Leu 91 (Figure 1d). The topology of the active site of PDF was similar in both β-sitosterol and ciprofloxacin, which is lined by interacting amino acids as predicted from the CASTp server and PDBSum (Figure 1e). β-sitosterol hydrogen bonded with the backbone hydrogen of Glu 133 of PDF with a bond distance of 2.128 and bond energy of -3.169 (Å units). Where as, in case of ciprofloxacin docking, the molecule was oriented at this site and the rest was inclined exhibiting hydrogen bond between terminal oxygen of Arg 97. The bond distance of backbone hydrogen of Glu 133 and oxygen of nitrofurazone radical was 1.959 and bond energy of -0.019 (Å units) (Figure 1f-1h). β-sitosterol has been proved to be one of the potent anti-bacterial agent which has been shown to elicit the *in-vitro* anti-bacterial activity better than the reference drug ciprofloxacin. By *in-silico* analysis, β-sitosterol showed that it can potentially inhibit the growth of bacteria by inhibiting PDF protein which plays an important role formylation-deformylation cycle essential for bacterial growth

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