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Research Article

IN VITRO AND *IN SILICO* APPROACHES ON THE ANTIBACTERIAL ACTIVITY OF *TINOSPORA CORDIFOLIA* METHANOLIC STEM EXTRACT

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ABSTRACT

Objective: The objective of the study was to evaluate the antibacterial activity of methanolic stem fraction of *Tinospora cordifolia* against *Escherichia coli* and *Staphylococcus aureus* by *in vitro* and *in silico* approaches.

Methods: In agar disc diffusion method, the inhibitory zone produced by various concentrations of the fraction showed a dose-dependent inhibition pattern. Minimum inhibitory concentration (MIC) values were calculated by broth dilution method. The total DNA present in the fraction treated bacterial cultures was estimated and compared with control DNA. The two-dimensional and three-dimensional structures of the gas chromatography–mass spectrometry (GC–MS) identified compounds were generated using ChemSketch tool. The docking studies were performed for analyzing the receptor and ligand interactions.

Results: The higher zone revealed the maximum inhibition of the growth of bacteria that were ranged from 2 mm to 6 mm for *E. coli* and 1.5 mm to 6.3±0.29 mm for *S. aureus.* MIC values showed that 30 µg/ml of the fraction was found as the effective dose. The DNA content isolated from the treated culture of both the strains was comparatively lesser than that of the untreated control culture. The GC–MS data analysis depicted the presence 15 major components in the fraction and the sharp peaks were obtained at time intervals 17.50, 20.27, 30.06, etc.

Conclusion: Thus, methanolic stem fraction of *T. cordifolia* possesses promising therapeutic activity against the urinary tract infection pathogens such as *E. coli* and *S. aureus* and a further exploration in the isolation and characterization such as plant-derived phytoconstituents would open up new ventures in the field of antibacterial drug discovery.

Keywords: *Tinospora cordifolia,* Minimum inhibitory concentration, DNA fragmentation, Gas chromatography–mass spectrometry/mass spectrometry, Molecular Docking.

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INTRODUCTION

Medicinal plants are considered to be new resources for producing agents that could act as alternatives to antibiotics in the treatment of antibiotic resistant bacteria. Phytotherapy is a science-based medicinal practice and thus distinguished from other, more traditional approaches, such as medical herbalism, which relies on an empirical appreciation of medicinal herbs and which is often linked to traditional knowledge [1]. The uropathogens such as *Escherichia coli* and *Staphylococcus aureus* develop into multidrug resistance organisms due to antibiotic resistance. They colonize in the bacterial epithelium through bacterial adhesions, leading to urinary tract infections (UTI). These microbes have acquired plasmid encoding for the extended spectrum β-lactamases responsible for antibiotic resistance [2]. A number of medicinal plants have been screened for antimicrobial activity in recent years and efforts are done to find their active constituents [3,4]. *Tinospora cordifolia* is commonly used in medicine where all the plant parts have curative property for certain diseases [5]. *T. cordifolia* exhibited properties such as anti-inflammatory, anti-allergic, anti-stress [6], antidiabetic, antispasmodic, and anticancer [7]. *T. cordifolia* has a wide array of bioactive principles such as Berberine, Choline, Tembetarine, Magnoflorine, Tinosporin, Palmetine, Isocolumbin, Aporphine alkaloids, Jatrorrhizine, Tetrahydropalmatine [8-10], Tinocordifolioside, Cordioside, Cordifolioside, Syringin, Cordifolioside A, B, C, D, and E [11,12], β-sitosterol, δ-sitosterol, and Giloinsterol [13,14]. *T. cordifolia* are used to treat various ailments such as fever, jaundice, diarrhea, dysentery, general debility, cough, asthma, leukorrhea, skin diseases, and fracture, bites of poisonous insects and venomous snakes and eye diseases. Thus, *T. cordifolia* has a better economic and therapeutic use.

METHODS

Chemicals and reagents

All chemicals and reagents used were of extra pure and culture grade, procured from suppliers such as Sigma Chemical Pvt. Ltd., USA; HiMedia Chemicals, Mumbai. All solvents were obtained from Fischer Scientific Ltd., India.

Collection of plant material

Collection of plant materials (*T. cordifolia* stem) was done in Tirunelveli district and Kodaikanal Hills, Tamil Nadu. Identification and authentication of the plant species were made at the Department of Botany, Holy Cross College, Trichy, Tamil Nadu. The collected plant specimen was prepared through the processes such as shade drying, chopping, and powdering. The coarse powder was later on used for solvent extraction process.

Preparation of plant extracts

The dried stem bark powder was weighed 2 kg to which 6 L of hexane was added and the mixture was shaken in an aspirator bottle occasionally for 72 h. This procedure was repeated 3 times and all extracts were decanted and pooled. Before drying, the extract was filtered using Whatman filter paper no. 2 on a Buchner funnel and then the solvent was removed by vacuum distillation in a rotary evaporator at 40°C; the extracts were placed in pre-weighed flasks before drying. A series of other solvent extractions on the plant residue, with ethyl acetate, methanol, and water, was sequentially done following the procedure as mentioned above [15].

Thin-layer chromatography (TLC)
The phytoconstituent detection and analysis were performed using TLC [16]. TLC supports the separation of the analyte between stationary phase (solid) and a mobile phase (liquid). Silica gel ($SiO_2 \times H_2O$) and alumina ($Al_2O_3 \times H_2O$) are commonly used solid phases in TLC. A pre-coated silica gel 60 F_{254}, 0.25 mm thick TLC plate (Merck) was loaded with 5 µl of a 100 mg sample/ml and kept dipped in a suitable solvent system. After the solvent run, visualization of the TLC plates was done under visible, ultraviolet (UV) light (254 and 360 nm, UV lamp) and iodine vapors and visible colored spots appear on the TLC plates. Plates were also sprayed with 5% sulfuric acid in ethanol and 0.36% sulfuric acid in methanol and heated for 3 min at 100°C to allow for the development of color changes [17].

Column chromatography
Column chromatographic technique was employed for the elution of active *T. cordifolia* stem methanol extract using a silica gel column (Acme's silica gel, 100–200 mesh size, 750 g, 3.5 i.d. ×60 cm) and the solvents were added in a specific order of hexane, ethyl acetate, and methanol solvent system (5%, 10%, 20%, 30%, 50%, 70%, and 100%) to polarity gradient elution. Finally, the column was washed with ethanol (100%). After chromatographic separation, a total of 128 fractions were obtained and each fraction was spotted on a pre-coated silica gel 60 F254, 0.25 mm thick TLC plate (Merck) and eluted in hexane:ethyl acetate (3:1), the Rf values were calculated for these fractions and the similar ones were pooled together. Rechromatographing of these fractions in a stepwise gradient of ethyl acetate:methanol (9:1) solvent system was performed with a result of getting 89 subfractions [18].

Gas chromatography–mass spectrometry (GC–MS)/MS
GC–MS analysis of these extracts was performed using ITQ900 Thermo Fisher Instrument, Holy Cross College, Trichy. The machine was run with fused silica capillary column. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 1 µl was employed. Injector temperature 250°C of ion source temperature 280°C was used. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C [19]. Interpretation on mass spectrum GC–MS was conducted using the database of National Institute Standard and Technology.

Bacterial cultures
Both Gram-positive (*E. coli*) and Gram-negative (*S. aureus*) bacteria causing secondary infections to diabetics were selected for the experiments. The bacterial cultures were obtained from the Department of Microbiology, K. A. P. V. Government Medical College, Tiruchirappalli, Tamil Nadu, India.

Antibacterial disc diffusion method
The antibacterial activity of the fractions eluted from *T. cordifolia* extract was tested *in vitro* using disc diffusion methods. A small aliquot (10 µl) of bacterial culture (*E. coli* and *S. aureus*) was transferred to the Mueller-Hinton agar plates aseptically [20]. The required sterile discs were placed on the agar medium. The prepared fraction was added to the discs in different concentrations and then incubated at 37°C for 24 h.

Determination of minimum inhibitory concentration (MIC)
MIC is the lowest concentration of extract that inhibits the growth of test organisms. Here, NCCLS [21] broth dilution method was used. Tetracycline (30 µg/ml) was used as a positive control in the antibacterial assays.

Statistical analysis
All data were expressed as mean±SEM for control and experimental groups. The data were analyzed using one-way analysis of variance on Statistical Package for the Social Sciences (Version 17.0) and the group means were compared by Duncan's multiple range test [22]. The results were considered statistically significant if the calculated "p"-value was less than 0.05.

DNA fragmentation
The control and extract treated (30 µg/ml) *E. coli* and *S. aureus* culture were incubated in the lysis buffer containing 9.34 ml TE buffer, 600 µl of 10% sodium dodecyl sulfate (SDS), and 60 µl of proteinase K (20 mg/ml) for 4 h at room temperature. Equal amounts of DNA were electrophoresed on 2.0% agarose gel and visualized in UV light [23].

Molecular docking studies
The structure of the compound was drawn using ChemSketch tool and the three-dimensional (3D) structure of the microbial receptor targets, autolysin, and alanine racemase was obtained from Protein Data Bank (PDB). The receptor proteins and ligands were prepared and docked using the LibDock module of Accelrys Discovery studio software 2.1. version to acquire the drug with protein interaction [24].

RESULTS

Antibacterial activity of methanolic stem extract of *T. cordifolia*
The antibacterial activity of the methanolic stem fraction of *T. cordifolia* on *E. coli* and *S. aureus* was evaluated by measuring the zone of inhibition in different concentrations (5, 10, 15, 20, 25, and 30 µg/ml) of the fraction and compared to the standard tetracycline disc (30 mg) (Figs. 1 and 2). The agar disc diffusion assay displayed a dose-dependent inhibition of bacterial growth with increasing concentrations of the fractions. A highest zone of inhibition was obtained for 30 µg/ml concentration of the fraction which ranged from 2 mm to 6 mm for *E. coli* and 1.5 mm to 6.3±0.29 mm for *S. aureus* (Table 1).

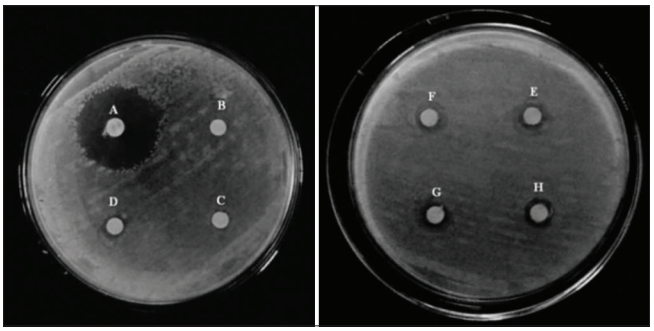

Fig. 1: Antibacterial activity of methanolic stem fraction of *Tinospora cordifolia* on *Escherichia coli* A – tetracycline (30 mg), B – control, C – 5 µg/ml, D – 10 µg/ml, E – 15 µg/ml, F – 20 µg/ml, G – 25 µg/ml, H – 30 µg/ml

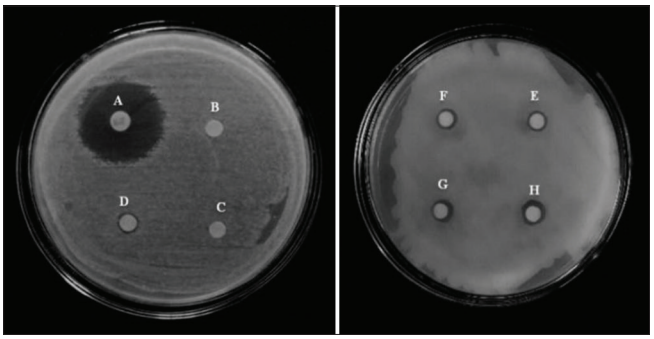

Fig. 2: Antibacterial activity of methanolic stem fraction of *Tinospora cordifolia* on *Staphylococcus aureus* A – tetracycline (30 mg), B – control, C – 5 µg/ml, D – 10 µg/ml, E – 15 µg/ml, F – 20 µg/ml, G – 25 µg/ml, H – 30 µg/ml

Bacterial growth inhibitory activity of methanolic stem extract of *T. cordifolia*

The MIC readings of methanolic stem fraction (30 µg/ml) of *T. cordifolia* for treated *E. coli* and *S. aureus* were observed and the result showed that maximum dilution of the fraction produced least inhibitory effect on growth of the bacteria with an increased optical density reading (Fig. 3).

Effect of methanolic stem extract of *T. cordifolia* against genomic DNA of bacteria

The bacterial cultures were treated with the standard concentration (30 µg/ml) of fraction followed by agarose gel electrophoretic analysis. When the DNA was analyzed, changes in its concentration and banding patterns were observed, both *E. coli* and *S. aureus* (Fig. 4). This depicts the suppression in DNA synthesis.

GC–MS/MS analysis

The GC–MS/MS result of the methanolic stem fraction of *T. cordifolia* reported the presence of 15 major components (Fig. 5). These components were identified with the peaks obtained in the GC graph, with the corresponding MS energy peaks and matching to the in-build database of library of compounds. Distinct, sharp peaks were obtained at time intervals 17.50, 20.27, 30.06, etc.

Docking studies

The structures of the compounds were drawn using ACD/ChemSketch tool and their 3D structures were generated (Fig. 6). The X-crystallographic structures of receptors autolysin and alanine racemase were retrieved from PDB using their PDB id. Then, protein molecules were prepared through the protein preparation wizard in the docking software, Discovery studio (version 2.1), and finally, the ligand is docked with receptor protein (Fig. 7). Compounds 1, 2, and 3 showed number of interactions and poses with LibDock scores 72.301, 90.613, 96.861, 110.007, 129.59, and 110.584 and absolute energies 12.395, 16.50, 15.868, 25.082, 39.8, and 52.241, respectively (Table 2). The molecular interactions including the H-bonds, amino acid residues, and atoms were analyzed.

DISCUSSION

Plants are the richest reserve of traditional drugs, serving as pharmaceutical intermediates and chemical entities for synthetic

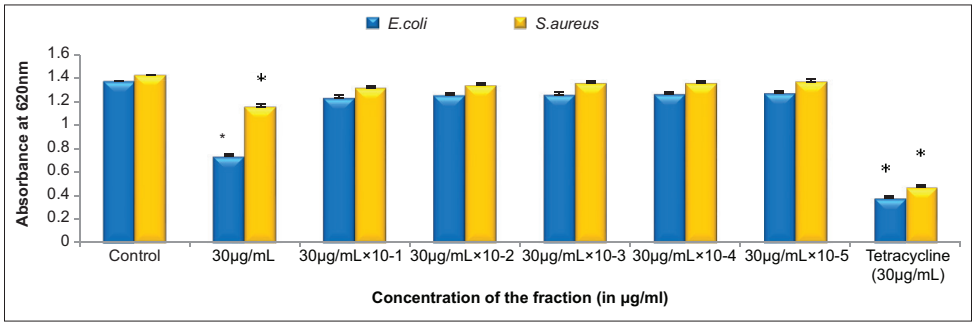

Fig. 3: Minimum inhibitory concentration estimated for methanolic stem fraction (30 µg/ml) of *Tinospora cordifolia* "*" represents statistical difference between control versus methanolic stem fraction of *T. cordifolia* treated organisms at significance value p<0.05

Table 1: Antimicrobial activity of methanolic stem fraction of *T. cordifolia* against bacterial pathogens

Microorganism	Zone of inhibition in mm*						
	Concentration of methanolic stem fraction of *T. cordifolia*						Tetracycline
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml	25 µg/ml	30 µg/ml	(30 µg/ml)
E. coli	-	2±0	3.3±0.29	4±0.58	5.5±0	6±0	15±1
S. aureus	-	1.5±0	3±0	4±0	5.3±0.58	6.3±0.29	19±1

*Each value represents mean±S.D. done in triplicates. *T. cordifolia: Tinospora cordifolia, E. coli: Escherichia coli, S. aureus: Staphylococcus aureus*

Table 2: Receptor-ligand interaction analysis

Compounds	Receptors	PDB ID	No. of poses	Absolute energy	LibDock score	No. of H-bond interactions	Bond length (Å)	Interacting residues
Compound 1	Autolysin	2B0P_A	75	12.395	72.301	1	2.0408	TYR204
	Alanine racemase	2RJG_A	47	16.50	90.613	1	2.274	LEU123
Compound 2	Autolysin	2B0P_A	86	15.868	96.861	3	2.4576	ASN303
							2.0365	TYR204
							2.2421	
	Alanine racemase	2RJG_A	17	25.082	110.007	1	2.4784	ARG209
Compound 3	Autolysin	2B0P_A	92	39.8	129.59	3	2.0741	ASP214
							2.1754	TYR204
							2.0927	ASN286
	Alanine racemase	2RJG_A	1	52.241	110.584	6	2.3807	GLY211
							2.1143	SER194
							2.0742	GLU221
							2.3742	PHE160
							2.2916	ALA163
							2.4686	ASP164

drugs [25]. Different parts of the plants such as root, stem, flower, fruit, and twigs produce biologically active constituents. *Tinospora* extracts are widely used in the traditional system of medicine to treat jaundice, rheumatism, urinary diseases, intermittent fevers, eye, and liver ailment. It is also an important constituent of many Ayurvedic formulations and immune modulatory activity in fighting infections [26]. The stem of *T. cordifolia* is a bitter stomachic, stimulates bile secretion, prevent vomiting and remedy for jaundice, diabetes, piles, respiratory disorders, neurological problems, and skin diseases. The antimicrobial potential of methanolic extract of *T. cordifolia* treated mice models reported an increased phagocytic activity of neutrophils against the bacterial pathogens [27]. The antibacterial potential of methanolic stem extract of *T. cordifolia* showed a better zone of inhibition as that of a similar to tetracycline for both *E. coli* and *S. aureus*. This inhibition is from the presence of alkaloid such as berberine in the extract that acts on higher binding affinity reversibly with ribosome to inhibit the bacterial protein synthesis [28]. It was a common observation that Gram-positive bacteria were more susceptible to plant extract as compared to Gram-negative bacteria. The antibacterial potential of methanolic stem extract of *T. cordifolia* by broth dilution method showed the lowest concentration of the compound that inhibits the highest concentration of the growth of bacteria that exert the broad-spectrum bacteriostatic activity of berberine by inhibiting the protein synthesis in bacteria [29]. In DNA synthesis, the extract showed its complete action on inhibiting the DNA synthesis for *E. coli* and *S. aureus* by the presence of more amount of berberine in the methanolic stem extract of *T. cordifolia* [30] causing alterations of the cytoplasmic membrane resulting in leakage of intracellular groups of nucleotides, amino acids, and the non-metabolizable sugar analog thiomethylgalactoside [31]. Therefore, the compound berberine in the extract was responsible for inhibition of DNA synthesis. The docking studies revealed the allosteric inhibitory action of the methanolic stem extract of *T. cordifolia* with higher binding affinity with autolysin and thus cell wall lysis by specifically hydrolyzing mucopeptide polymers in the bacterial cell wall [32].

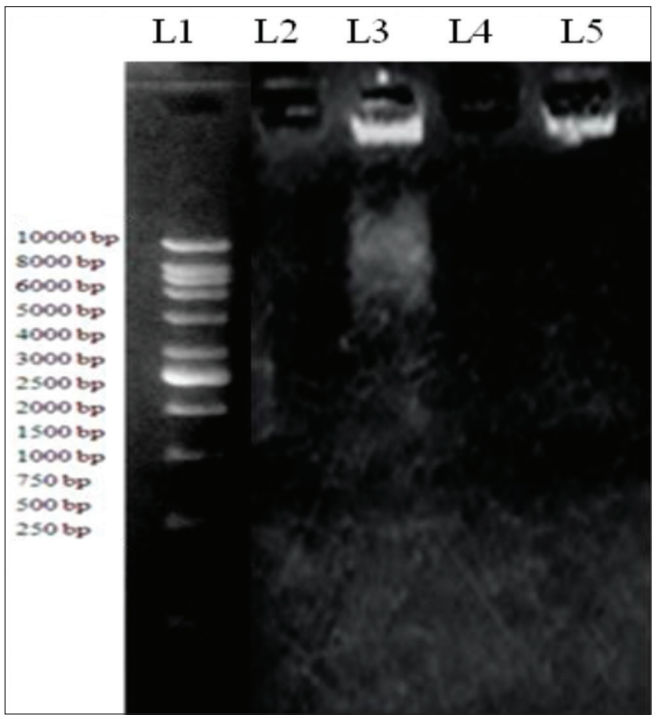

Fig. 4: DNA analysis methanolic stem fraction (30 µg/ml) of *Tinospora cordifolia* on *Staphylococcus aureus*. Lane 1 – DNA marker, lane 2 – control *S. aureus*, lane 4 – methanol fraction treated *S. aureus*

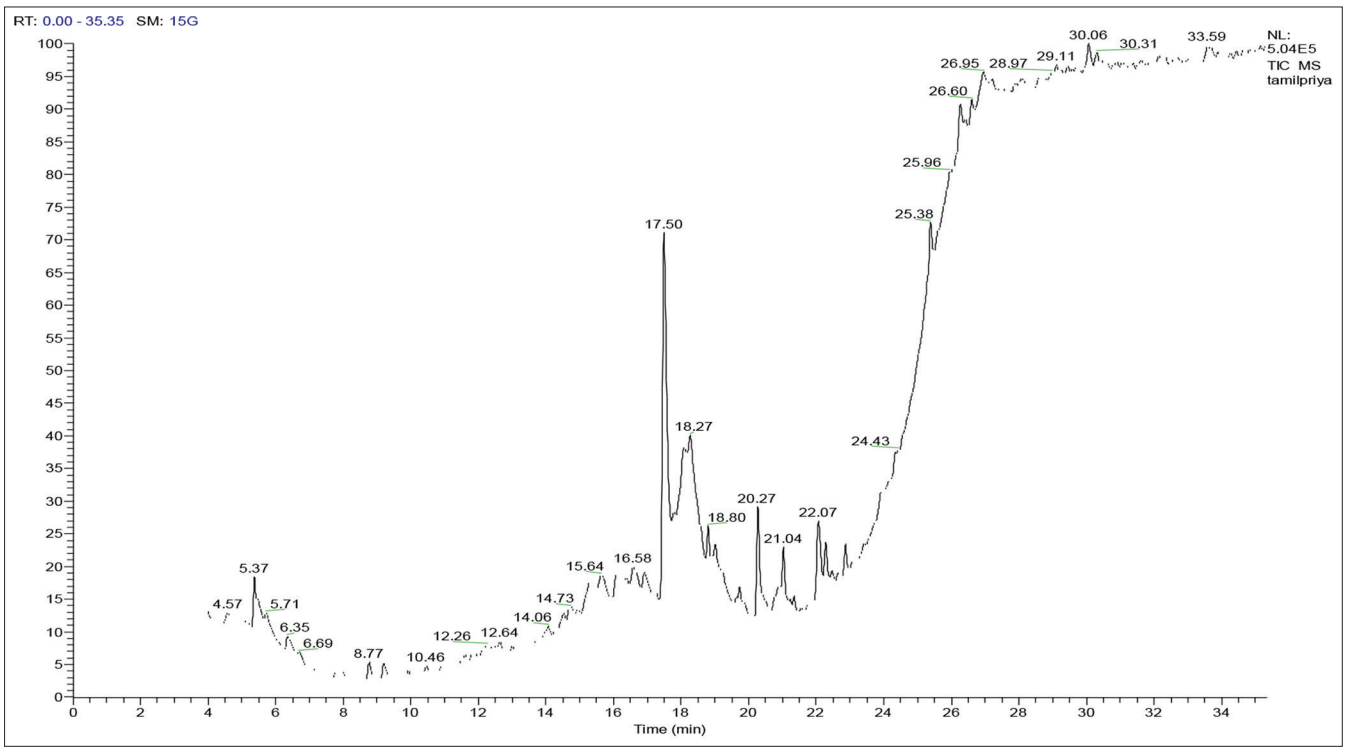

Fig. 5: Gas chromatography–mass spectrometry (MS)/MS analysis of methanolic stem fraction of *Tinospora cordifolia*

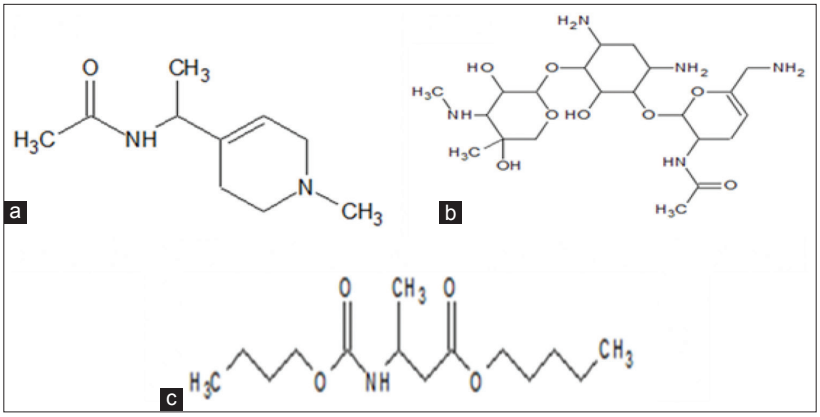

Fig. 6: Structure of compounds. (a) – Compound 1, (b) – Compound 2, (c) – Compound 3

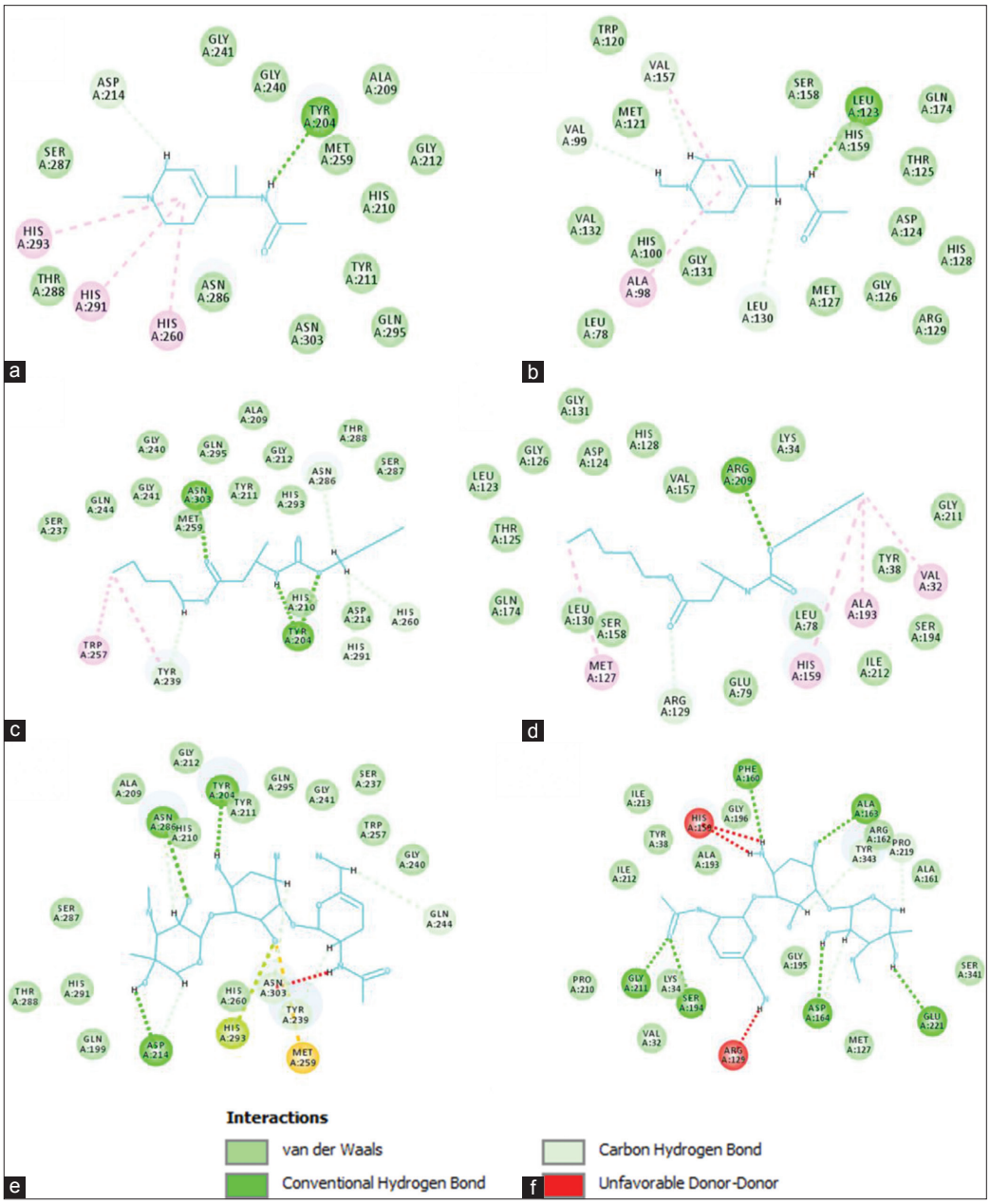

Fig. 7: Receptor-ligand interaction analysis through docking studies. a) Interaction between compound 1 and autolysin. b) Interaction between compound 1 and alanine racemase. c) Interaction between compound 2 and autolysin. d) Interaction between compound 2 and alanine racemase. e) Interaction between compound 3 and autolysin. f) Interaction between compound 3 and alanine racemase

CONCLUSION

Thus, methanolic stem fraction of *T. cordifolia* possesses promising therapeutic activity against the UTI pathogens such as *E. coli* and *S. aureus* and a further exploration in the isolation and characterization such as plant-derived phytoconstituents would open up new ventures in the field of antibacterial drug discovery.

ACKNOWLEDGMENT

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AUTHORS' CONTRIBUTIONS

Ms. Thamizhpriya did all *in vitro* studies, experimental work, and writing the article. Dr. Avila Jerley did acquisition of data and manuscript editing. Dr. Shalini Gnanam did acquisition of data and manuscript editing. Ms. Jeyakani did molecular docking studies and manuscript writing. Ms. Indu did GC–MS/MS analysis and manuscript writing. Dr. Rajalakshmi did research guidance and critical revision of manuscript.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest.

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