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Research Article
Antibacterial and Molecular Docking Studies of Bioactive Component from Leaves of *Stachytarpheta cayennensis* (Rich.) Vahl.

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Abstract

Background: In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. The use of plant compounds to treat infections is an age-old practice in developing countries, where there is dependence on traditional medicine for a variety of diseases. Interest in plants with antimicrobial properties has revived as a result of current problems associated with the use of antibiotics. **Materials and Methods:** This study is designed to isolate phytoconstituent 3, 4, 4a, 5, 8, 8a-hexahydro-6-methylisochromen-1-one (HMIC) from leaves extract of *Stachytarpheta cayennensis* and test the antibacterial activity against different pathogenic bacterial species and *in silico* glucosamine-6-phosphate synthase (GlcN-6-P) inhibition property of the HMIC. The phytoconstituents HMIC was isolated from the crude ethanolic extract and purification was carried out by column chromatography using silica gel (100-200 mesh size) and n-hexane-ethyl acetate (7:3) as eluting system, the compound was characterized by analytical ^{1}HNMR, ^{13}C NMR, IR and mass spectral data. The antibacterial activity of HMIC was evaluated against Gram-positive and Gram-negative bacteria using the agar-well diffusion method and automated docking was used to determine the orientation of inhibitors bound in the active site of GlcN-6-P synthase employing AutoDock 3.0. **Results:** The phytoconstituent HMIC showed the strongest antibacterial activity against *Klebsiella pneumoniae* followed by *Pseudomonas aeruginosa* where as it showed moderate activity on *Staphyllococcus aureus* of the bacterial growth. It also possesses better glucosamine-6-phosphate synthase inhibition in molecular docking studies with minimum docking and binding energy and better ligand efficiency when compared to standard. **Conclusion:** This compound was isolated for the first time from this plant and no evidence could be found for the previous reported presence of HMIC in the genus *Stachytarpheta*. Considering the antibacterial activity, this could offer a scientific basis for the therapeutic potency of *Stachytarpheta cayennensis* used in traditional medicine. Further studies are necessary to determine the toxicity, side effects, circulating levels, pharmacokinetic properties, diffusion in different body sites and the mechanism involved with the antibacterial activity of HMIC.

Key words: Antibacterial, docking, ethnomedicine, glucosamine-6-phosphate synthase, medicinal plant

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stachytarpheta cayennensis belongs to the family Verbenaceae, commonly known as "Kaadu uttaraani" is an important plant having therapeutic value, they have been shown to possess immune boosting activity[1], antiulcer activity[2], antidiarrheal activity[3], anti-inflammatory and analgesic activity[4], antipyretic and hepatoprotective activity[5-7].

The plant leaves also finds folkloric usage in the treatment of helminthiasis, constipation, hypertension, diabetes, stomachic, febrifuge, chronic liver diseases, flues, cough, arthritis, diuretic and sudorific[8,9].

The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, the attempts to characterize these properties in the laboratory date back to the early 1900s[10,11].

As plant-derived medicines have made a large contribution to human health and well-being since ancient times, plants have provided a source of inspiration for novel drug compounds. It is essential to study medicinal plants which have folklore reputation to promote the proper use of herbal medicine and to determine their potential as sources for new drugs[12-14]. Over the past few years, many efforts have been made to discover new antimicrobial compounds from various kinds of natural sources. In this regard several Indian medicinal plants have been evaluated and a fair number possess potential antimicrobial activity[15]. Among those few products have been approved as new antibacterial drugs[16,17]. However, due to the extensive use of antibiotics there is a increased prevalence of antibiotic resistant bacteria which are making current antimicrobial agents insufficient to control some bacterial diseases. Therefore, study for identifying novel substances that are active against human pathogens is an urgent need[18].

The antimicrobial properties of plants have been investigated by a number of studies and many of them have been used as therapeutic alternatives[19]. Thus, searching not only for improved versions of existing drugs but also for new drug targets has become an urgent need. The key enzyme L-glutamine: D-fructose-6-phosphate amidotransferase, also known as glucosamine-6-phosphate synthase (EC 2.6.1.16) is responsible for the synthesis of glucosamine-6-phosphate (GlcN-6-P) from D-fructose-6-phosphate and L-glutamine. This enzyme is first in the pathway leading to the formation of UDP-N-acetylglucosamine (UDP-GlcNAc), a product that is present in all types of organisms, but is used by these organisms in different ways[20,21]. It is used to build macromolecules important for the cell wall assembly, such as chitin, mannoproteins and peptidoglycans in prokaryotes. In mammals, UDP-GlcNAc is utilised for biosynthesis of glycoproteins and mucopolysaccharides[22]. In spite of the fact that glucosamine-6-phosphate synthase is present in all kinds of cells, it may be exploited as a target for potential antimicrobial drugs and selective toxicity can be achieved[23]. Glucosamine-6-phosphate, the product of this enzyme is indispensable for microbes as well as for human cells, yet the consequences of its deficiency in both species are very different. It has been shown that even a short-time inactivation of GlcN-6-P synthase in bacteria is lethal for the pathogen by inducing morphological changes, agglutination and lysis, while in mammals depletion of the aminosugar pool for a short time is not lethal, because of the much longer lifespan of mammalian cells, long half lifetime of GlcN-6-P synthase and rapid expression of the mammalian gene encoding this enzyme[24-26].

The objective of this study was to isolate and investigate the antibacterial effects of 3, 4, 4a, 5, 8, 8a-hexahydro-6-methylisochromen-1-one, a molecule from traditionally proven plant *Stachytarpheta cayennensis* and compare the mode of interactions existing through *in silico* study, in the hunt of better therapies against microbial diseases and provide scientific evidence to folkloric claim of the plant.

MATERIALS AND METHODS

Plant material: Fresh leaf materials of plants in the flowering stage were collected in and around the Kuvempu University Campus, Karnataka (Southern India) in May, 2012. The taxonomic identification of the plant was confirmed by Dr. L.B. Chaudhary, Scientist, National Botanical Research Institute, Lucknow (Voucher specimen No. 249092 (LWG)).

Extraction and isolation: Freshly collected leaf material of *Stachytarpheta cayennensis* were shade-dried and then powdered using a mechanical grinder. The pulverized plant material were taken in one liter capacity thimble of Soxhlet apparatus and refluxed with ethanol (LR grade, Merck, India) until all soluble compounds had been extracted in 2 batches of 500 g each. Extraction was considered to be complete when the filtrate had a faint colour. The extract was evaporated to dryness (yield: 38.6%) under reduced pressure using a Rotavapor (Buchi Flawil, Switzerland). Thus obtained crude extract was dissolved in ethanol and adsorbed on silica gel powder and loaded on a silica gel column (Merck, 100-200 mesh size). The column was eluted by mixtures of n-hexane-ethyl acetate in the ratio of 7:3. Various fractions were collected and concentrated to obtain the compound (yield:0.18%). Eluted compound was characterized

with the help of NMR (^{1}HNMR and ^{13}CNMR), mass and IR spectroscopy. The isolated compound was then subjected for the evaluation of antibacterial activity.

Bacterial culture: The bacterial strains used in this study were clinical isolates from different infection status of patients presenting symptoms of *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* associated diseases. The isolates were identified by a standard method[27]. The standard strains used were *Klebsiella pneumoniae* (MTCC-618), *Pseudomonas aeruginosa* (ATCC-20852) and *Staphylococcus aureus* (ATCC-29737). The organisms were maintained on nutrient agar slope at 4°C and sub-cultured into nutrient broth by a picking-off technique[28] for 24 h before use.

Bacterial susceptibility testing: The antibacterial activity of isolated compound was studied against Gram-negative and Gram-positive bacteria by the agar well diffusion method[29]. Nutrient agar (Hi Media, India) was used as the bacteriological medium. The isolated compound was dissolved in 10% aqueous dimethylsulfoxide (DMSO) to a final concentration of 100 µg/100 µL. Pure DMSO was taken as the negative control and 50 µg/100 µL ciprofloxacin as the positive control.

One hundred microliters of inoculum was aseptically introduced on to the surface of sterile agar plates and sterilized cotton swabs were used for even distribution of the inoculum. Wells were prepared in the agar plates using a sterile cork borer of 6.0 mm diameter. One hundred microliters of test and control compound was introduced in the well. The same procedure was used for all the strains. The plates were incubated aerobically at 35°C and examined after 24 h[30,31]. The diameter of the zone of inhibition produced by each agent were measured with a ruler and compared with those produced by the commercial antibiotic ciprofloxacin.

Molecular docking studies: Automated docking was used to determine the orientation of inhibitors bound in the active site of GlcN-6-P synthase as target for antibacterial activity. A Lamarckian genetic algorithm method, implemented in the program AutoDock 3.0 was employed. The ligand molecules HMIC and ciprofloxacin were designed and the structure was analyzed by using ChemDraw Ultra 6.0. The 3D coordinates were prepared using PRODRG server[32]. The protein structure file (PDB ID: 1XFF) was taken from PDB (www.rcsb.org/pdb) was edited by removing the heteroatoms, adding C terminal oxygen[33]. For docking calculations, Gasteigere-Marsili partial charges[34] were assigned to the ligands and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The grid map was centered at particular residues of the protein which was predicted from the ligplot and were generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-solis and wets methods were applied for minimization, using default parameters[35].

Statistical analysis: The results of the experiment are expressed as Mean±SE of three replicates in each test. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple pairwise comparison tests to assess the statistical significance. The $p<0.05$ was considered as statistically significant, using software ezANOVA version 0.98.

RESULTS AND DISCUSSION

The ethanol extract was subjected to column chromatography to furnish orangish red colored waxy mass. The IR spectra shows the presence of a ester group by exhibiting an absorption band at 1736 cm^{-1}, a C=C group due to the presence of a band at 1639 cm^{-1} and C-O by the band at 1024 cm^{-1}. In the ^{1}H NMR spectrums the signal at δ 5.20 shows the presence of one unsaturated proton. The signal at δ 3.6 indicates the presence of protons attached to oxygen function. The bunch of signals in-between 0.9-1.1 indicates the presence of methyl and methylene groups. The signal at δ 2.00 indicates the presence of protons adjacent to carbonyl groups. The ^{13}C NMR spectrum shows strong signals at δ 14.09 for a methyl group, δ 171.06 for a carbonyl group, the pair of signals at 133.93 and 139.13 indicates the presence of a C=C group, at δ 60.30 indicating the carbon attached to a oxygen function and the other signals at δ 20.89, 22.60, 29.60, 31.85 and 33.75 indicates the presence of carbons atoms of methylene and methane groups. The molecular ion peak at m/z166 indicated that the molecular weight of the compound is 166. The melting point of the compound was recorded on electrothermal melting point apparatus and observed melting point was 78-80°C. The molecular weight in conjunction with ^{13}C NMR and ^{1}H NMR analysis data led to the assignment of molecular formula as $C_{10}H_{14}O_2$. Based on spectral data the compound is characterized as 3, 4, 4a, 5, 8, 8a-hexahydro-6-methylisochromen-1-one (Fig. 1).

The antibacterial activity of HMIC was examined with ciprofloxacin a well known broad spectrum antibacterial agent. In the agar diffusion method, the HMIC emerged as active agent against *K. pneumoniae* and *P. aeruginosa* where as it showed moderate activity against *S. aureus*. The results obtained for the activity is presented in Table 1.

Klebsiella pneumoniae and *Pseudomonas aeruginosa* were the opportunistic pathogens that cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia and a variety of systemic infections. The *Staphylococcus aureus* causes a variety of suppurative (pus forming) infections and toxinoses in humans. It also causes superficial skin lesions such as boils and also more serious infections such as pneumonia, mastitis, phlebitis and meningitis. Reports indicated that clinical isolates from different infectious sources from hospitals showed resistance against the drug methicillin[36,37]. The search for new antimicrobial agents is an important line of research because of the resistance to drugs acquired by the microorganisms.

Table 1: Antibacterial activity of HMIC against various bacterial strains by agar well diffusion method

Bacterial strains	HMIC	Ciprofloxacin
Klebsiella pneumoniae	12.73±0.18	23.00±0.12
Klebsiella pneumoniae (MTCC-618)	12.00±0.12	21.87±0.47
Pseudomonas aeruginosa	11.00±1.00	22.93±0.58
Pseudomonas aeruginosa (ATCC-20852)	7.00±0.58	20.53±0.79
Staphyllococcus aureus	7.67±0.33	19.67±0.33
Staphyllococcus aureus (ATCC- 29737)	4.87±0.44	18.33±0.67

Values are the mean of three experiments Mean±SE

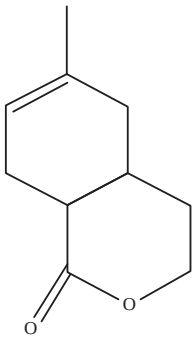

Fig. 1: Structure of 3, 4, 4a, 5, 8, 8a-hexahydro-6-methylisochromen-1-one

The results of this investigation revealed that the HMIC showed the potent antibacterial activity against the clinical strains of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from different infectious sources. Among all, Gram-negative bacteria, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were more susceptible to HMIC than Gram-positive bacterium *Staphylococcus aureus.* This observation contradicts the earlier reports that plant extracts are more active against Gram-positive bacteria than Gram-negative bacteria[38,39].

This could be attributed to the fact that the cell wall in Gram-positive bacteria has a single layer, whereas, the Gram-negative cell wall is a multi-layered structure[40], acting as a barrier to many environmental substances, including antibiotics[41]. But their activity is probably due to their ability to react with extracellular and soluble proteins and to complex with bacterial cell walls[42].

The docking of HMIC with glutamine amido transferase domain reveals that, our compound exhibited interactions with one or the other amino acids in the active pocket (Fig. 2). The docking results for HMIC and ciprofloxacin are

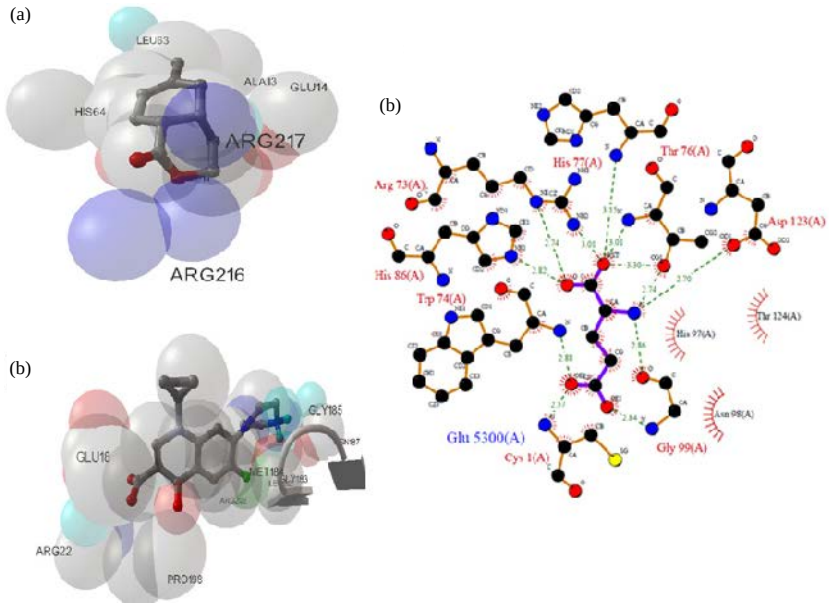

Fig. 2(a-c): (a) Orientation of HMIC in the active pocket of GlcN-6-P synthase, (b) Enfolding of ciprofloxacin in active pocket and (c) Interacting amino acids as predicted from the ligplot

Table 2: Molecular docking results with glucosamine-6-phosphate synthase

Molecule	Binding energy	Docking energy	Inhibitory constant	Intermol energy	H-bonds	Bonding
HMIC	-7.29	-7.29	4.51e-006	-7.29	3	HMIC::DRG1:OAH:GPS:A:PRO198:O HMIC::DRG1:OAB:GPS:B:THR200:HG1 HMIC::DRG1:OAB:GPS:B:ARG201:HN
Ciprofloxacin	-10.26	-10.79	3.02e-008	-11.19	2	CF::DRG1:OAB:GPS:A:ARG201:HN CF::DRG1:OAA:GPS:B:ARG201:HE

documented in Table 2. Practically, HMIC showed good docking energy and ligand efficiency compared to standard. The HMIC was completely enfolded in the entire active pocket of GlcN-6-P synthase (Fig. 2a) as compared to ciprofloxacin (Fig. 2b). The topology of the active site of GlcN-6-P synthase was similar in both HMIC and standard, which is lined by interacting amino acids as predicted from the ligplot (Fig. 2c). The earlier investigations[43] noticed that the catalytic nucleophile in glutaminase domain of bacterial glucosamine-6-phosphate synthase and the nucleophilic character of its thiol group appears to be increased through general base activation by its own alpha-amino group. Similar results are also obtained by Vidya *et al.*[35] where they have used plant derived compound as ligand for antibacterial docking studies. By *in silico* analysis, it seems that HMIC is promoting the remarkable antibacterial activity through the inhibition of GlcN-6-P synthase. Hence, HMIC has been proved to be one of the potent antibacterial agent.

This study therefore confirms the bactericidal nature of HMIC with its ability to suppress *S. aureus*, *K. pneumoniae* and *P. aeruginosa* and it could be used as cheap, safe and effective alternative to synthetic counterpart in the management of bacterial infections.

CONCLUSION

The present results offer a scientific basis for the therapeutic potency of *Stachytarpheta cayennensis* used in traditional medicine. However, the activity level of the isolated compound may be more accurately evaluated in terms of MIC values as the zone of inhibition might be influenced by solubility and diffusion rate of the phytocompounds. In addition, *in vivo* studies are necessary to determine the toxicity of the active constituents, their side effects, circulating levels, pharmacokinetic properties and diffusion in different body sites. Since, the isolated molecule has shown the better activity profile against gram negative and gram positive bacteria, it is a best target for further research for the development of broad spectrum antibacterial agents.

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