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RESEARCH ARTICLE

Structural Characterization and Antimicrobial Activity of *Cocculus hirsutus L.* Leaf Extract by Liquid Chromatography-mass Spectroscopy

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ABSTRACT

Many number of the plant species including *Cocculus hirsutus L.* is being used as the sources of herbal medicine. Present work was mainly focused with the identification of the therapeutic properties of *C. hirsutus L.* leaf extracts. The leaf extracts of methanol, aqueous, chloroform, and benzene showed solvent dependent qualitative and quantitative phytochemical presence as well as antimicrobial activity. Whereas the leaf extracts of methanol and chloroform showed significantly high antimicrobial activity than water and benzene extracts. Further methanol leaf extract of *C. hirsutus* performed to liquid chromato y-mass spectroscopy (LC-MS) for identification of active antimicrobial compound structure. LC-MS studies give 26 structural compounds. Docking (annotating) studies revealed that among 26 compounds the Compound-5 **(**Hexadecanoic acid - (1R, 2R, 3S, 4R, 6S)-4, 6-diamino-2, 3-dihydroxy cyclohexyl 2,6-diamino-2,6-dideoxy-α-D-glucopyranoside) showed highest docking fitness score with the bacterial membrane protein sortase-A. Our data suggest that methanol extract of *C. hirsutus* leaf possess medicinally significant antimicrobial compounds and thus justify the use of this leaf as folklore medicine for preventing human microbial related diseases.

Keywords: Antimicrobial activity, *Cocculus hirsutus* L., docking studies, phytochemical screening, sortase-A.

INTRODUCTION

Medicinal plants are resources of new drugs; it is estimated that there are more than 2, 50,000 flowering plant species. *Cocculus hirsutus L.* belongs to the family Menispermaceae; it is a tropical, invasive creeper, native to India, Pakistan, and tropical Africa. It is having wide medicinal properties such as skin infection, dhieria, anticancer, eczema, rheumatism, and gonorrhea.[1,2] Many of the modern medicines are produced indirectly from medicinal plants, for example, aspirin. Plants are directly used as medicines by a majority of cultures around the world, for example, Chinese medicine and Indian medicine, for example, garlic.[3]

Usually, plant extracts occur combination of several bioactive or phytochemical compounds with different polarities; their separation still remains a big challenge for the process of identification and characterization of bioactive compounds.[4] It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as thin-layer chromatography, column chromatography, flash chromatography, Sephadex chromatography, and high-performance liquid chromatography (HPLC) should be used to obtain pure compounds.[5] The pure compounds are then used for the determination of structure and biological activity. Molecular docking technique is one of the most interesting tools applied in pharmaceutical research to determine the structure of bioactive compounds.[6] In this method, one can dock small molecules into receptor targets which help to predict ligand conformation and orientation within a targeted binding site.[7,8] The main objective of the present study is to characterized medicinal importance phytochemical compounds against common disease-causing microorganisms from leaf extracts of *C. hirsutus L.* by liquid

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chromatography-mass spectroscopy (LC-MS) and docking studies.

MATERIALS AND METHODS

Collection of plant material

The plant samples of *C. hirsutus* were collected from the surrounding field areas of Rayalaseema University campus, Kurnool district, Andhra Pradesh. The leaves were washed thoroughly with tap water followed with sterilized distilled water for the removal of dust and sand particles, then shade dried at room temperature. Samples were homogenized to a fine powder by a mechanical grinder and used for experimental analysis.

Preparation of plant extracts

5 g of leaf sample powder were sequentially extracted with solvents, namely methanol, chloroform, and benzene and also with water by Soxhlet apparatus for 48 h. Then, it was filtered through Whatman No.1 filter paper. The crude extracts were obtained by dissolving a known amount of dry extract in different solvents such as methanol, chloroform, benzene, and aqueous to obtain a stock solution of 1000 µg/ml. The stock solutions were serially diluted with the respective solvents to obtain lower dilutions (50, 25, and 10 µg/ml). Crude extracts were analyzed for phytochemical and antimicrobial studies.

Preliminary phytochemical analysis

The individual extracts were subjected to qualitative chemical investigation for the identification of different phytochemical compounds and plant secondary metabolites using standard protocols followed by Mayer's and Wagner's procedures.

Antibacterial and antifungal activity

The method called agar well plate method was adapted for the screening of antimicrobial study. The hot sterile medium was poured into the sterile Petri plates to form a 2–3 mm thick. The plates were lawn cultured with bacterial broth and fungal spore suspension. Then make a hole with a diameter of 6–8 mm is punched aseptically with a sterile tip, and then poured a volume 150 µl of crude extract at desired concentrations (50, 25, and 10 µg) were introduced into each well. Then, agar plates were incubated under ambient conditions in an incubator at 37°C for 24 h depending on the test organisms. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested, then evaluated the inhibition zones.

Preparation of standard solutions

Ciprofloxacin was the positive reference (control) standard with the dilutions of 50, 25, and 10 µg/mL.

Test organisms (bacterial stains)

The microorganisms used in the present study include bacteria such as *Pseudomonas*, *Klebsiallea pneumonia* and *bacillus subtilis*. All bacterial were procured from biotechnology lab, S.K. University, Anantapur. The bacterial and fungal stock cultures were maintained on different nutrient media which were stored at 4°C.

Quantification of polyphenols by LC-MS/MS

The phytochemical were analyzed by the chromatographic system consisting of an Agilent 1100 series HPLC instrument equipped with 6460 triple quad MS detector.[9] The methanol leaf extracts of *C. hirsutus* were carried out by LC-MS for getting their active structure. We prepared working solutions with a concentration of 10, 25, 50, and 100 µg/ml. The internal standard stock solution, 1, 7-diaminoheptan (C7H18N2) was also prepared from 10 to 100/ml. Then, we added different volumes of perchloric acid to obtain a final volume of 0.5 ml. Quantitative measurements were performed depending on the internal standard, using the chromatography peaks obtained for each biogenic amine. The absorbance of derivatives compounds was measured at 270 nm, and the peaks were integrated with ChromQuest software. Each compound concentration was expressed in µg/ml, and the compound content was expressed in mg/kg. The results obtained are of 10 determinations; the mean values were calculated with Microsoft Excel software. Then, active site of all derived structural compounds was determined by docking program compared with sortase-A protein (PDB ID: 1IJA), it was obtained from the protein data bank (http://www.rcsb.org/pdb).

Molecular docking

Docking was performed with AutoDock genetic optimization of ligand docking (GOLD) software which is based on genetic algorithm. To analyze the effect of ligand association, all the water molecules and the heteroatoms have been removed from the target protein. All the hydrogen atoms were added to the protein as it is required for the electrostatics and then nonpolar hydrogen atoms were merged together. Finally, removal of unnecessary chains and hetero atoms using SPDBV software, hydrogens were added to the protein and used for active site identification.

RESULTS AND DISCUSSION

Preliminary qualitative and quantitative phytochemical studies

We analyzed qualitative and quantitative analysis of different phytochemical compounds in leaf extracts of *C. hirsutus* using the Soxhlet apparatus with different solvents (methanol, chloroform, benzene, and water). It was observed that methanol, chloroform, and benzene extracts of leaf crude extract were shown more phytochemical presence such as flavonoids, steroids, alkaloids, tannins, glycosides, and lignins in methanol, chloroform, and benzene extracts than water extracts, respectively, results were represented in Table 1. Similar results were reported by Nayak and Singhai, Akroum and Lalaoui, and Ravichandra *et al.*[10-12] and their reports stated that methanol, chloroform, and ethanol extracts show more phytochemical than water extracts.

Antimicrobial activity

We measured the antimicrobial activity of *C. hirsutus* leaf extracts in different solvent extracts on common bacterial and fungal species. The disc diffusion method was used to determine the zone inhibition range. Efficiency of crude drug on different microorganisms was calculated based on the inhibition zone in diameter (mm), results were represented in Figure 1. Aqueous, methanol, ethanol, and benzene extracts were showed significant activity against *Pseudomonas*, *Klebsiella pneumoniae* in Gram-negative, and *Bacillus subtilis* in Gram-positive at the concentration of 50 µg. Whereas aqueous, methanol, and ethanol extracts show maximum inhibition activity and benzene extract showed less antimicrobial activity. Our results correlated with earlier reports[13] and the ethanol extracts of *Tectona grandis* show maximum bacterial inhibition.

Phytochemical structure by LC-MS

We analyzed 26 different phytochemical structures by LC-MS from leaf methanol extract of *C. hirsutus* results were presented in Table 2.

Docking analysis

A total of 26 phytochemical compounds were performed to docking analysis to predict different active sites (ligand-receptor interaction and also to rank the compounds based on the binding energies or fitness score) with known bacterial protein sortase-A. Docking studies revealed that among 26 compounds, only 8 compounds were

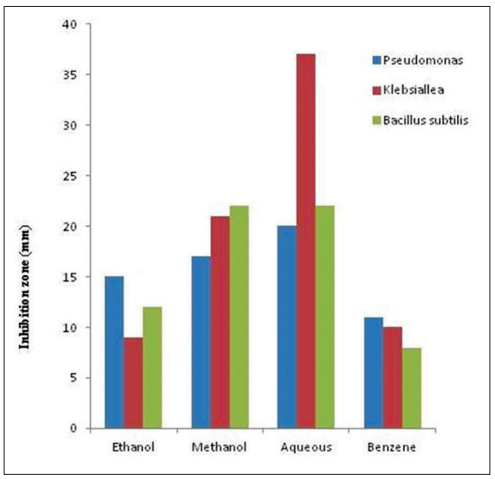


Figure 1: Diameter of the zone of inhibition (mm) of different leaf extracts of *C. hirsutus* L. against different pathogenic bacterial strains

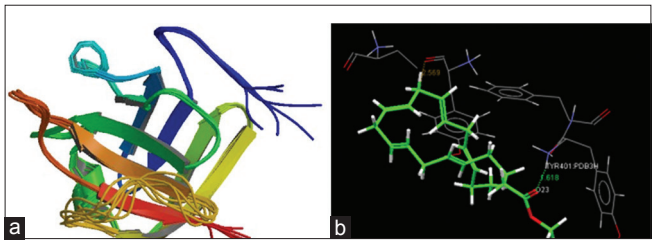


Figure 2: (a) Structure of sortase-A, (b) docking structure of hexadecanoic acid - (1R, 2R, 3S, 4R, 6S)-4,6-diamino-2,3-dihydroxycyclohexyl 2,6-diamino-2,6-dideoxy-α-D-glucopyranoside with sortase-A

Table 1: Preliminary phytochemical screening of *C. hirsutus L.* leaf extracts

S. No	Compounds	Methanol	Aqueous	Chloroform	Benzene
1.	Alkaloids	+	_	+	_
2.	Flavonoids	+	+	+	+
3.	Phenols	_	_	_	_
4.	Glycosides	_	_	+	+
5.	Tannins	+	_	+	_
6.	Steroids	+	_	+	+
7.	Quinones	_	_	_	_
8.	Lignin's	+	_	+	_
9.	Saponins	_	_	_	_
10.	Fixed oils	_	_	_	_

(+): Presence of phytochemical compound, (-): Absence of phytochemical compound. *C. hirsutus: Cocculus hirsutus*

Table 2: Active phytochemical compounds of *C. hirsutus* by LC-MS

S.No.	Compound Label-CH	RT	Mass	MFG formula	MFG diff (ppm)
1.	Cpd 86: C17 H39 N3 O5	20.641	365.2891	C17 H39 N3 O5	-0.43
2.	Cpd 87: C17 H30 N2 O4	20.76	326.2209	C17 H30 N2 O4	-1.1
3.	Cpd 88: C21 H44 N4 O10	20.996	512.3055	C21 H44 N4 O10	0.38
4.	Cpd 89: C17 H30 N2 O4	21.05	326.2209	C17 H30 N2 O4	-0.9
5.	Cpd 92: C28 H58 N4 O8	22.398	578.4245	C28 H58 N4 O8	1.69
6.	Cpd 93: C20 H40 N O9	22.746	438.2697	C20 H40 N O9	1.33
7.	Cpd 94: C19 H28 N2 O6	22.807	380.1943	C19 H28 N2 O6	1.16
8.	Cpd 95: C17 H26 N5 O5	23.074	380.1947	C17 H26 N5 O5	-3.54
9.	Cpd 96: C33 H40 N2 O7 S	23.77	608.2553	C33 H40 N2 O7 S	0.51
10.	Cpd 100: C32 H62 N4 O3 S	24.32	582.4558	C32 H62 N4 O3 S	-2.6
11.	Cpd 103: C29 H40 N2 O12	24.643	608.2573	C29 H40 N2 O12	1.3
12.	Cpd 104: C19 H34 N2 O4	24.962	354.2523	C19 H34 N2 O4	-1.37
13.	Cpd 105: C20 H36 N2 O4	25.137	368.2677	C20 H36 N2 O4	-0.58
14.	Cpd 106: C19 H34 N2 O4	25.261	354.2522	C19 H34 N2 O4	-0.96
15.	Cpd 107: C26 H38 N4 O13	25.381	614.2434	C26 H38 N4 O13	0.21
16.	Cpd 108: C29 H40 N2 O11	25.426	592.2625	C29 H40 N2 O11	1.21
17.	Cpd 109: C20 H36 N2 O4	25.437	368.2672	C20 H36 N2 O4	0.73
18.	Cpd 111: C21 H38 N2 O4	25.782	382.2834	C21 H38 N2 O4	-0.63
19.	Cpd 112: C33 H60 N O4	25.965	534.4568	C33 H60 N O4	-8.47
20.	Cpd 113: C27 H38 N5 O10	26.164	592.2617	C27 H38 N5 O10	0.26
21.	Cpd 115: C40 H62 N2 O	26.432	586.4875	C40 H62 N2 O	-2.26
22.	Cpd 121: C22 H45 N3 O4	27.473	415.3414	C22 H45 N3 O4	-1.02
23.	Cpd 126: C24 H44 N2 O4	27.892	424.33	C24 H44 N2 O4	0.28
24.	Cpd 127: C25 H46 N2 O4	28.351	438.345	C25 H46 N2 O4	1.63
25.	Cpd 128: C23 H28 O5	28.691	384.1933	C23 H28 O5	0.87
26.	Cpd 129: C30 H48 N2 O5	28.848	516.3559	C30 H48 N2 O5	0.78

C. hirsutus: Cocculus hirsutus, LCMS: Liquid chromatography-mass spectroscopy

showed maximum fitness score with targeted protein sortase-A. Among these 8 compounds, the 5 (Cpd 92: hexadecanoic acid - (1R, 2R, 3S, 4R, 6S)-4,6-diamino-2,3-dihydroxy cyclohexyl 2,6-diamino-2,6-dideoxy-α-D-glucopyranoside**)** showed highest gold fitness score with sortase-A [Figure 2a and b] [Tables 3 and 4].

DISCUSSION

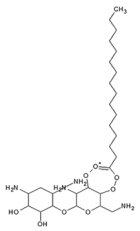
Plants have long been a very important source of drug and many plants have been screened whether they contain compounds with therapeutic activity. Therefore, it is vital to evaluate the antimicrobial activity of *C. hirsutus*. Medicinal plants play an

Table 3: Gold fitness score of *C. hirsutus* all compounds against active site of sortase-A

S.No	Fitness	S (hb_ext)	S (vdw_ext)	S (hb_int)	S (int)	Ligand name
1.	20.05	7.32	18.11	0.00	-12.18	86
2.	-15.31	6.00	27.64	0.00	-59.32	87
3.	12.32	8.15	20.09	0.00	-23.46	88
4.	-16.84	0.00	15.30	0.00	-37.89	89
5.	23.89	0.30	22.77	0.00	-7.72	92
6.	-2.16	5.59	13.15	0.00	-25.83	93
7.	-37.47	0.00	11.60	0.00	-53.43	94
8.	-44.87	7.47	10.35	0.00	-66.58	95

C. hirsutus: Cocculus hirsutus

Table 4: Structural evaluation of compound 5 (Cpd: 92)

Compound label-CH	RT	Mass	Name	MFG formula	MFG diff (ppm)	Structure
Cpd 92: C28 H58 N4 O8	22.398	578.4245	Hexadecanoic acid -1R,2R,3S,4R,6S)-4,6-diamino-2, 3-dihydroxycyclohexyl2,6-diamino-2, 6-dideoxy-α-D-glucopyranoside	C28 H58 N4 O8	1.69	

RT: Retention time

important role and constitute the backbone of traditional medicine. According to the World Health Organization estimate, 80% of populations in developing countries rely exclusively on traditional medicine for their health care need. Moreover, 20% of the available allopathic drugs have an active principal obtained from higher plants.[14] Plant synthesizes natural products as its chemical weapon that arrests the growth of environmental microbes,[15] and some plants inhibit the growth of potential human pathogens too. In the present study, phytochemical compounds of *C. hirsutus* showed solvent, concentration-dependent antimicrobial activity significantly. Docking studies prescribed that the *C. hirsutus* having a significant antimicrobial compounds.

CONCLUSION

Phytochemical compounds of *C. hirsutus* extracts were identified by LC-MS and characterization studies performed by docking by GOLD3.0.1 software. *Cocculus hirsupus* extracts generate almost 26 active compounds. Among 26 compounds, the compound hexadecanoic acid - (1R, 2R, 3S, 4R, and 6S) -4, 6-diamino-2, 3-dihydroxy cyclohexyl 2,6-diamino-2,6-dideoxy-α-D-glucopyranoside showed maximum fitness score with bacterial protein sortase-A. It shows *Cocculus hirsutus* methanol leaf extract having high antimicrobial activity than other solvent extracts.

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