

Evaluation of antimicrobial efficacy of methanolic extract of *Cocculus hirsutus* (L.) Diels

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Context: The persistent increase in the number of antibiotic resistant strains of microorganisms has led to the development of more potent but more expensive antibiotics. Synthetic drugs are mostly associated with side effects and are generally costly, hence are not affordable to economically poor class of the society when long-term treatment is required, thus interest has been developed in the use of herbal medicines which have been reported to have either very little or no side effects. **Aims:** Present work was carried out to assess the antimicrobial activity of crude methanolic extract of *Cocculus hirsutus* against some multidrug resistant pathogenic bacteria. **Materials and Methods:** Different plant parts of *C. hirsutus* were collected and air dried and soxhlet extracted using methanol as solvent. These extracts were then tested for antimicrobial activity using agar-well diffusion method. Inhibition zone, activity index, minimum inhibitory concentration were also calculated. **Statistical Analysis:** Mean value and standard deviation were calculated for each test bacteria and fungus. Data were analysed by one-way ANOVA and *P* values were considered significant at *P* < 0.05. **Results:** Maximum Zone of inhibition was observed in the callus extracts against *Staphylococcus epidermidis* (3.9 ± 0.56 mm) amongst the bacteria species and against *Phanerochaete chrysosporium* (2.5 ± 0.63 mm) amongst the fungal species. Methanolic extracts of leaf and stem showed varied activity with a different strain of bacteria and fungi. Methanolic extract of leaf and stem showed maximum inhibition zone against *Micromonospora* sp. bacterial strain. In this study, methanolic extract of callus sample showed highest promising minimal inhibitory concentration of 61.1 µg/ml in *S. epidermidis*. **Conclusion:** Results of the present study reveal that methanolic extracts of *C. hirsutus* are showing great antimicrobial potential against tested microorganisms and may be exploited for future antimicrobial drugs.

Key words: Activity index, *Cocculus hirsutus*, inhibition zone, methanolic extract, minimum inhibitory concentration

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an enormous number of modern drugs have been isolated from natural sources especially from plants; many of these isolations are based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care.^[1] The study of medicinal plants has attracted many researchers, owing to the useful applications of plants for the treatment of various diseases in humans and animals. Infectious diseases emanating from microorganisms such as bacteria, fungi, viruses, and parasites are a major threat to public health care due to the growing resistance of many microorganisms to currently available antibiotics. The incidence of fungal infections

has increased dramatically over the past few decades,^[2] mainly affecting immunocompromised or surgically treated patients as well as the young and old.^[3] With the rise in infections caused by various fungi and the development of resistance in fungal pathogens, it is important that novel antifungal agents be identified and developed.^[4] Recently scientific interest in medicinal plants has burgeoned due to the increased efficiency of the plant derived drugs and raising concern about the side effects of modern medicine. The rising prevalence of microorganism showing resistance to antibiotics has urged mankind to develop new antimicrobial compounds. Being non-toxic and easily affordable, there has been a resurgence in the consumption and demand for medicinal plants.^[5]

The roots and leaves of *Cocculus hirsutus* have great medicinal value and are used both, internally as well as externally for medicinal purpose. Root is bitter and used as alterative, laxative, demulcent, tonic, diuretic, antiperiodic in fever, in malaria, joint pains, in the treatment of skin diseases constipation and kidney problems.^[6] Juice of leaves coagulates in water and forms mucilage which is used externally as a cooling medicine in eye problems and soothing application in prurigo, ecsema, impetigo, and dyspepsia. When juice is sweetened with sugar, it is given in acute gonorrhoea.

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Decoctions of the root is mixed with long pepper is used in chronic rheumatism and syphilitic cachexia.^[7] Knowing the ethnobotanical and pharmacological applications of the plant, the main objective of this research was to assess antimicrobial activity of leaf, stem and callus samples of *C. hirsutus* against human pathogens. To screen for biological activity, the crude methanolic extract was prepared and tested against eight different microorganisms (four bacteria and four fungi). The aim of screening was to correlate and confirm the antimicrobial activity to the traditional uses of plants. This can be seen as a step in the search for primary health care products that are socially acceptable and scientifically valuable.

MATERIALS AND METHODS

Collection of Plant Material

Plant parts of *C. hirsutus* were collected from Kulish Smriti Van, Jaipur and specimen were compared with the voucher specimen available at Herbarium of Department of Botany, University of Rajasthan, Jaipur. The fresh plant samples (*C. hirsutus*: leaf and stem) were collected and washed individually under running tap water to remove soil particles and other dirt. Furthermore, *in-vitro* callus obtained on Murashige and Skoog, medium fortified with indole-3-acetic acid (0.5 mg/l) was also taken for the present study.

Preparation of Extract

The *in vivo* leaf and stem were dried in the laboratory at room temperature for 7 days while the callus was dried at 60°C for 2 days in an oven. All dried samples were ground well into a fine powder in a mixer grinder. The powder was extracted by soxhlet extraction method using methanol as solvent. Later, the solvent was distilled under reduced pressure in a rotary vacuum evaporator until the extracts became dry. The crude evaporated plant extracts were dried at room temperature for 5–30 days. Then 50 mg of each crude plant extract was dissolved in 1 ml (1,000 µl) of the solvent to give a final concentration of crude extract in solvent of 50 mg/ml. Then, this extract was used for antimicrobial assay.

Test Microorganisms

Eight different strains of microorganisms were used in the screening process viz. (1) *Zymomonas mobilis* microbial type culture collection and Gene Bank (MTCC 88), (2) *Staphylococcus epidermidis* (MTCC 3615), (3) *Staphylococcus aureus* (MTCC 3160), (4) *Micromonospora* sp. (MTCC 3296) and antifungal screening against (1) *Alternaria solani* (MTCC 2101), (2) *Fusarium culmorum* (MTCC 349), (3) *Phanerochaete chrysosporium* (MTCC 787), (4) *Penicillium chrysogenum* (MTCC 161) collected from the MTCC, Institute of Microbial Technology, Chandigarh, India. The bacteria were grown in nutrient broth (Himedia, M002) at 37°C

and maintained on nutrient agar slants at 4°C and fungal cultures were grown and maintained on potato dextrose agar slants at 4°C.

Antimicrobial Activity

Antimicrobial assay of the crude extracts was performed against eight tested pathogenic strains by agar well diffusion method. The bacterial strains were grown on nutrient agar medium (agar 15 g, beef extract 3 g, sodium chloride 5 g and peptone 5 g, in one litre distilled water) at 37°C for 18 h and were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 MacFarland standards (108 CFU/ml). The suspension was used to inoculate 90 mm diameter petri plates. Wells (6 mm diameter) were punched in the agar and filled with the test samples (crude methanol extracts of flower and leaf samples of plant) to get different concentrations viz. 15, 25, 35, 45, 55 µl of the extract. Ampicillin was used as a standard for anti-bacterial assay and fluconazole for antifungal assay. Plates were incubated at 37°C ± 2°C for 24 h. Antimicrobial activities were evaluated by measuring the inhibition zone diameters, and the activity index was calculated for each of these. The experiments were conducted in triplicate. The same method was followed for testing antifungal activity using potato dextrose agar medium.

$$\text{Activity index} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

Determination of Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of various extracts against tested microorganisms was determined by broth dilution method. For broth dilution, 1 ml of a standardised suspension of strain (10⁶ CFU/ml) was added to each tube containing extracts at various concentrations in nutrient broth medium. The tubes were incubated at 37°C for 24 h (for bacterial strains) and 25°C for 48 h (for fungal strains) and observed for visible growth after vortexing them gently. The MIC is taken as the lowest concentration of the extracts at which there is turbidity after incubation. The highest dilution of a plant extract that still retains an inhibitory effect against the growth of microorganisms is known as MIC.

Statistical Analysis

Mean value and standard deviation were calculated for each test bacteria and fungus. Data were analysed by one-way ANOVA, and *P* values were considered significant at *P* < 0.05.

RESULTS

The antimicrobial activity of methanolic extract of leaf, stem (*in vivo*) and callus (*in vitro*) against bacteria and fungi examined in the present study and its potency was

quantitatively assessed by the presence or absence of inhibition zones and zone diameters.

The results of the antimicrobial activity are presented in Table 1 [Figures 1 and 2]. Antimicrobial screening of methanolic extract from leaf, stem and callus of *C. hirsutus* revealed that the methanolic extract of callus showed better antimicrobial activity against micro-organisms as compared to methanolic extract of leaf and stem part. Maximum zone of inhibition was obtained with

Table 1: Antimicrobial activity of methanolic extract of leaf, stem and callus of *C. hirsutus*

		IZ/AI	Standard	Leaf	Stem	Callus
Microorganisms						
Bacteria						
<i>Z. mobilis</i>	IZ	4.0±0.37		1.8±0.54	1.3±0.43	2.4±0.73
	AI			0.45	0.325	0.6
<i>S. aureus</i>	IZ	3.4±0.93		2.0±0.29	1.6±0.32	3.1±0.67
	AI			0.588	0.470	0.911
<i>S. epidermidis</i>	IZ	5.8±0.66		2.1±0.69	2.3±0.62	3.9±0.56
	AI			0.362	0.396	0.672
<i>micromonospora</i> sp.	IZ	4.1±0.78		2.3±0.78	2.5±0.48	3.8±0.77
	AI			0.56	0.609	0.926
Fungi						
<i>A. solani</i>	IZ	2.2±0.37		1.7±0.44	1.4±0.54	2.0±0.59
	AI			0.772	0.77	0.909
<i>F. culmorum</i>	IZ	2.9±0.67		1.9±0.73	2.1±0.47	2.3±0.59
	AI			0.655	0.724	0.793
<i>P. chrysosporium</i>	IZ	2.1±0.63		1.8±0.58	1.5±0.33	2.5±0.63
	AI			0.857	0.714	1.19
<i>P. chrysogenum</i>	IZ	1.9±0.68		1.3±0.59	1.6±0.69	1.8±0.65
	AI			0.684	0.842	0.947

C. hirsutus – *Cocculus hirsutus*; *Z. mobilis* – *Zymomonas mobilis*; *S. aureus* – *Staphylococcus aureus*; *S. epidermidis* – *Staphylococcus epidermidis*; *A. solani* – *Alternaria solani*; *F. culmorum* – *Fusarium culmorum*; *P. chrysosporium* – *Phanerochaete chrysosporium*; *P. chrysogenum* – *Penicillium chrysogenum*; IZ – Inhibition zone; AI – Activity index

methanolic extract of callus as compared with leaf and stem. Maximum zone of inhibition was observed in the callus extracts against *S. epidermidis* (3.9 ± 0.56 mm) [Table 1, Figure 1c] amongst the bacteria species and against *P. chrysosporium* (2.5 ± 0.63 mm) [Table 1, Figure 2b] amongst the fungal species. Methanolic extracts of leaf and stem showed varied activity with a different strain of bacteria and fungi. Methanolic extract of leaf and stem showed maximum inhibition zone against *Micromonospora* sp. bacterial strain. Zone of inhibition obtained with leaf extract was 2.3 ± 0.78 mm and stem extract was 2.5 ± 0.48 mm while in case of fungal strain maximum inhibition zone was observed against *F. culmorum*. Zone of inhibition obtained with leaf extract was 1.9 ± 0.73 mm, and stem extract was 2.1 ± 0.47 mm.

The MIC method was used to further investigate extracts that showed broad spectrum activity against microorganisms. The highest dilution of a plant extract that still retained an inhibitory effect against the growth of microorganisms (absence of the zone of inhibition) was reported as the MIC. In this study, methanolic extract of callus sample showed highest promising MIC of 61.1 µg/ml in *S. epidermidis* [Table 2]. Results of MIC value of different plant parts are summarised in Table 2. MIC of the stem extract for different micro-organisms ranged between 17.8 and 45.5 µg/ml while that of the leaf extract ranged between 20 and 59.3 µg/ml [Table 2]. The MIC of ampicillin and fluconazole control ranged between 19 and 33.21 µg/ml.

DISCUSSION

Antimicrobial are compounds that at low concentrations exert an action against microorganism and exhibit therapeutic toxicity towards them.^[8] These can be any substances of natural, synthetic or semi-synthetic origin that may be used to kill microorganisms including bacteria, fungi, and viruses.^[9] There is a continuous and urgent need

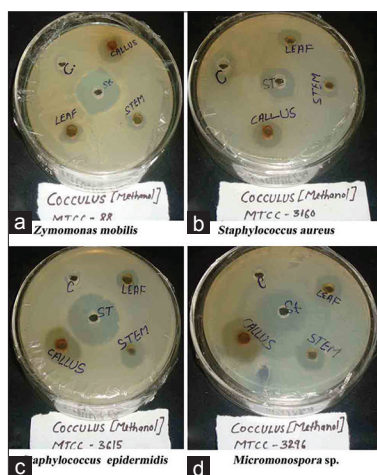


Figure 1: (a-d) Antibacterial activity of methanolic extract leaf, stem, and callus of *Cocculus hirsutus*

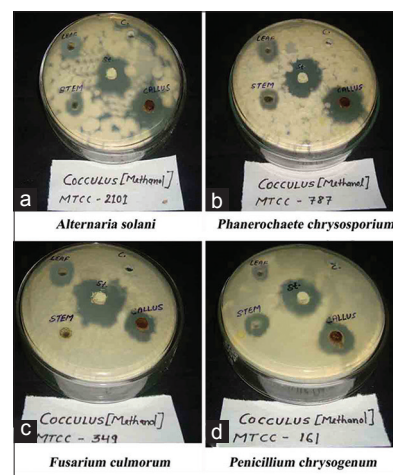


Figure 2: (a-d) Antifungal activity of methanolic extract of leaf, stem and callus of *Cocculus hirsutus*

Table 2: MIC value of methanolic extract of leaf, stem and callus of *C. hirsutus*

	Microorganism (MIC (µg/ml))			
	Standard	Leaf	Stem	Callus
Bacteria				
<i>Z. mobilis</i>	30.6	55.1	41.3	49.8
<i>S. aureus</i>	29.3	45.3	31.4	33.7
<i>S. epidermidis</i>	33.21	59.3	45.5	61.1
<i>Micromonospora</i> sp.	23.7	30	22.3	36.4
Fungi				
<i>A. solani</i>	19	20	17.8	27.1
<i>F. culmorum</i>	20.8	30.2	29.8	26
<i>P. chrysosporium</i>	21.2	23	20.5	30.2
<i>P. chrysogenum</i>	28.4	26.5	28	31.5

C. hirsutus – *Cocculus hirsutus*; *Z. mobilis* – *Zymomonas mobilis*; *S. aureus* – *Staphylococcus aureus*; *S. epidermidis* – *Staphylococcus epidermidis*; *A. solani* – *Alternaria solani*; *F. culmorum* – *Fusarium culmorum*; *P. chrysosporium* – *Phanerochaete chrysosporium*; *P. chrysogenum* – *Penicillium chrysogenum*; MIC – Minimum inhibitory concentration

to discover new antimicrobial compounds as there is an alarming increase in the incidence of new and re-emerging infectious diseases. Medicinal plants may be a viable alternatives source to costly antibiotics (against which microbes are developing resistance rapidly), as most of the medicinal plants are safe with little or no side effects, cost effective and have ability to affect a wide range of antibiotic resistant microorganisms.

Present study is an effort towards this direction. *C. hirsutus* had previously been studied for antibacterial and antifungal activities, but still the literature available is meagre. Hence, an attempt was made to identify the antimicrobial activity of crude methanolic extract of *C. hirsutus* against eight different microorganisms (four bacteria and four fungi). Mostly the crude extracts have been screened of the whole aerial part, without MIC, minimum bactericidal concentration, and total activity determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence cannot replace the existing antibiotics. In the present investigation inhibition zone, activity index, MIC have been evaluated for each extract. The aim of this study was to correlate and confirm the antimicrobial activity to the traditional uses of plants. Antimicrobial screening of methanolic extract from leaf, stem and callus of *C. hirsutus* revealed that the methanolic extract of callus showed better antimicrobial activity against micro-organisms as compared to methanolic extract of leaf and stem part. Maximum zone of inhibition was obtained with methanolic extract of callus as compared with leaf and stem.

Maximum zone of inhibition was observed in the callus extracts against *S. epidermidis* (3.9 ± 0.56 mm) amongst the bacteria species and against *P. chrysosporium* (2.5 ± 0.63 mm) amongst the fungal species. Methanolic extracts of leaf

and stem showed varied activity with a different strain of bacteria and fungi. Methanolic extract of leaf and stem showed maximum inhibition zone against *Micromonospora* sp. bacterial strain. Zone of inhibition obtained with leaf extract was 2.3 ± 0.78 mm and stem extract was 2.5 ± 0.48 mm while in case of fungal strain maximum inhibition zone was observed against *F. culmorum*. Zone of inhibition obtained with leaf extract was 1.9 ± 0.73 mm, and stem extract was 2.1 ± 0.47 mm. In this study, methanolic extract of callus sample showed highest promising MIC of 61.1 µg/ml in *S. epidermidis*. The MIC of the stem extract for different micro-organisms ranged between 17.8 and 45.5 µg/ml while that of the leaf extract ranged between 20 and 59.3 µg/ml.

Among bacterial pathogens, gram-positive bacterial strains were found to be more susceptible than gram-negative bacterial strains. This may be attributed to the fact that cell wall in gram-positive bacteria consists of a single layer, whereas, gram-negative cell wall is a multi-layered structure bounded by an outer cell membrane. Similar results were reported earlier by Chanda and Baravalia.^[10]

The cidal activities of medicinal plants are due to the active constituents present in them. It is also proved that *C. hirsutus* contains certain constituents like alkaloids, glycosides, flavonoids and tannins which are antibiotic principles of this plant. These antibiotic principles are actually the defensive mechanism of the plants against different pathogens,^[11] which enables the extract to overcome the barrier in Gram-negative cell wall,^[12] and decreases the bacterial proliferation by blocking key enzymes at microbial metabolism.^[13] From the results obtained in this study, it is evident that the methanolic extract of different samples of *C. hirsutus* is effective against all the tested pathogens. Methanol was found to be a good solvent system for the extraction of the total phenolic compounds. Similar results were obtained in *Machilus odoratissima*^[14] and *Echinophora platyloba*.^[15] An important aspect comprises the search for new compounds that have antimicrobial action and synergism with currently available antimicrobial drugs, since bacteria resistant to conventional medicines are increasingly frequent; consequently, medicinal plants constitute an alternative for infection treatment.

The antimicrobial activity of plants was proven by various examples, in the form of both essential oils and extracts. Thus, this property can be a promising ally in the development of medicines necessary to combat the increasing number of bacterial strains that become resistant to conventional antibiotics. This work may provide essential information in the selection of plant extract for further isolation of constituents responsible for the activity against the studied species, thereby aiding to explore an

antibacterial lead that is helpful in combating the diseases caused by micro-organisms.

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