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Original Article

PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTIMICROBIAL ACTIVITY OF *CALOTROPIS GIGANTEA, LAWSONIA INERMIS* AND *TRIGONELLA FOECUM- GRAECUM*

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ABSTRACT

Objective: The aim of the present study was to screen phytochemicals and antimicrobial activities of the extracts of three medicinal plants viz. *Calotropis gigantea, Lawsonia inermis* and *Trigonella foenum-graecum*.

Methods: The methanolic extracts of the three plants were tested against clinical isolates of five bacterial strains (*Staphylococcus aureus, Bacillus cereus, Methicillin Resistant Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*) and three fungal strains (*Candida albicans Trichophyton rubrum* and *Aspergillus flavus*) using agar well diffusion method and Minimum Inhibition Concentration (MIC) by broth micro dilution.

Results: *L.inermis* showed maximum antimicrobial activity against all the tested bacteria and fungi compared to the other plants. Preliminary phytochemical screening revealed the presence of alkaloids, tannins, flavonoids, terpenoids, cardiac glycosides, saponins and steroids. The bioautographic results revealed that the maximum zone of inhibition of *L.inermis* extract was observed at R_f 0.28 and 0.22 for flavonoids and tannins respectively.

Conclusion: The results of present study indicated that flavonoids and tannins are the major bioactive compounds which show potent antimicrobial activity against pathogenic microorganisms.

Keywords: Medicinal plants, Phytochemical, Antimicrobial activity, Clinical isolates, Bioautography.

INTRODUCTION

Historically, medicinal plants have provided the basic building blocks for a number of highly effective drugs and they remain as an attractive option for discovery of new molecular entities, due to their largely untapped chemical diversity [1]. Nearly, 80% of the world's population relies on traditional medicines for primary health care, mostly involving plant extracts. In India, almost 95% of the prescriptions were based on traditional systems of Unani, Ayurveda, Homeopathy and Siddha [2]. The study of plants continues principally for the discovery of novel secondary metabolites. *C.gigantea* is a waste land weed, better known as milkweed belonging to the family Apocynaceae and habitat of Asian countries. Locally it is used to cure several illnesses such as toothache, ear-ache, sprain, anxiety, pain, epilepsy, diarrhea and mental disorders. *C.gigantea* exhibited anticandida activity, cytotoxic activity, antipyretic activity and wound healing activity [3].*L.inermis* is a flowering plant belongs to the family Lythraceae which resulted in the production of bioactive compound, lawsone. *L.inermis* is used as a cooling agent, astringent, antifungal and antibacterial herb for the skin and scalp. It has also been used as a dye and preservative for hair, skin and fingernails as well as leather and clothes [4].*T.foenum-graecum* is a medicinal plant which belongs to the family Fabaceae, being rich in phytochemicals and has traditionally been used as a food, forage, and therapeutic effects. *T.foenum-graecum* is used as antimicrobial for the skin and scalp diseases [5]. Despite abundant literature on antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for chemical use as antibiotics. A significant part of the chemical diversity produced by plants is to protect against microbial pathogens. Hence, they have been proven to have antimicrobial importance both *in vitro* and *in vivo* [6].There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity [7].

MATERIALS AND METHODS

Plant collection and preparation for extraction:

The plant samples *C. gigantea L.inermis* and *T. foenum-graecum* were collected locally in Bangalore, Karnataka, India. For the analysis of antimicrobial activity *C. gigantea* latex, *L.inermis* leaves and *T. foenum-graecum* seeds were used for study.

Preparation of plant extracts

The fresh latex of *C. gigantea* was aseptically collected from the aerial parts of the healthy plant [8]. in to clean glass tubes containing double distilled water. The latex mixture was kept overnight at 4°C, supernatant was selectively discarded and centrifuged at 10000 rpm for 10 min at 4°C. The pellet was collected and kept in cool place for further use. *L. inermis* (leaves) and *T.foenum graecum* **(**seed) clean with tap water and followed by distilled water and shade dried. Dried leaves and seeds were powdered and stored in dry and cool place for further use [9].

Extraction procedure

The prepared samples extracted with Soxhlet extractor at room temperature methanol as solvent (70 % V/V) and extraction process repeated 4 cycles. The extracts were filtered through Whatman No.1 filter paper. The filtrate was concentrated by using rotary evaporator under reduced pressure at 60°C.

Phytochemical screening

The extracts were subjected to phytochemical analysis to ascertain the presence metabolites such as alkaloids, tannins, flavonoids, terpenoids, cardiac glycosides, saponins and steroidsusing standard procedures [10].

Antimicrobial susceptibility test:

i) Bacterial and Fungal strains and growth medium:

The bacterial strains (*Staphylococcus aureus, Bacillus cereus, Methicillin Resistant Staphylococcus aureus, Pseudomonas aeruginosa,*

and *Escherichia coli*) and fungal strains (*Candida albicans, Trichophyton rubrum* and *Aspergillus flavus*) were clinically isolated. The isolated strains were sub cultured on Luria Bertani (LB) and Sabouraud dextrose agar (SDA) for bacterial and fungal strains. All these cultures were maintained at 4°C for further use.

ii) Antimicrobial activity

The modified agar well diffusion method of Perez *et al* [11] was employed. LB agar and SDA were used for bacteria and fungi respectively. Once the agar was solidified, 50µl of the different bacterial and fungal cultures were spread onto the plates using a sterile glass spreader. The plates were punched with five millimeter diameter wells and filled with 25µl of the different concentrations of 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml and 2.0mg/ml plants extracts of and blanks (distilled water which served as the negative control). Simultaneously, Amoxicillin (10µg/ml) and Amphotericin B (10µg/ml) were used as positive controls for bacteria and fungi respectively. The tests were carried out in triplicates. The bacterial plates were incubated at 37°C for 24 hrs, and fungal plates at room temperature. The diameter of the zone of inhibition was measured in millimeters at 24 hrs and 72 hrs for bacteria and fungi respectively.

iii) Minimum Inhibitory Concentration (MIC) of *L.inermis*

As the *L.inermis* exhibited prominent control over the growth of the bacteria and fungi, MIC was determined for *L.inermis* against bacteria and fungi through broth dilution method. The stock solution was prepared to make a concentration range from 1 to 50 mg/ml. The concentration of test cultures was adjusted to 0.5 McFarland standards. The 100 µl of bacterial suspension was added to the sterile screw. All the tubes were incubated at 37°C for 24hrs and they were examined for visible turbidity. The MIC values were identified as the lowest concentration that inhibited the visible growth of the tested bacteria.For fungi MIC was determined by using broth micro dilution method according to standard protocols. All the tubes were incubated at 37°C for 72hrs and they were examined for visible turbidity [9].

Thin layer chromatography:

The *L.inermis* extracts which showed best antimicrobial activity against *B.cereus* and *A.flavus* were analyzed using thin layer chromatography (TLC).About 15 µl of extract was applied to the precoated aluminum silica gel 60 F, Merck F_{254}. Developing solvent system used was toluene and ethyl acetate (1:1 v/v).The separated spots were visualized under UV light. The plates were developed according to Johann *et al* [12] for the identification of active phytoconstituents.

Bioautographic Analysis:

For bio autographic analysis, developed TLC plates were dried overnight and sprayed with a concentration of suspension of actively growing *B.cereus* and *A.flavus.* The plates were incubated at 37°C in a chamber at 100% relative humidity. Subsequently, plates were sprayed with a 2 mg /ml solution of MTT (3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) and further incubated for 4 h. Microbial growth appeared as clear zones against pink back ground. The R_f values of the spots showing were determined [13].

Statistical analysis

All the experiments were carried out in triplicates. Results were expressed as the mean ± S.E of three independent experiments (n=3).

RESULTS AND DISCUSSION

Methanolic extracts of three medicinal plants (*C.gigantea, L.inermis* and *T.foenum-graecum*) were screened for antimicrobial activity against five clinical isolates of bacteria (*Staphylococcus aureus, Bacillus cereus, Methicillin Resistant Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*) and three fungi (*Candida albicans Trichophyton rubrum* and *Aspergillus flavus*). Results of phytochemicals screening (Table 1) revealed the presence of alkaloids, tannins, flavonoids, saponins, terpenoids, steroids, and cardiac glycosides.

Table 1: Phytochemical screening of methanolic extracts of medicinal plants

Phytochemicals	Plants extracts		
	C.gigantea	*L.inermis*	*T.foenum-graecum*
Alkaloids	+	+	+
Cardiac glycosides	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Steroids	+	+	+
Tannins	+	+	+
Terpenoids	+	+	+

+ = Positive, - = Negative

L. inermis showed best antimicrobial activity as compared to *C. gigantea* and *T.foenum-graecum*.The different concentrations of *L.inermis* extract showed zone of inhibition against *B.cereus*(9.2±0.13 mm to 20.3±0.19 mm) and against *A.flavus*(10.3±0.15 mm to 21.3±0.24 mm), these results are in accordance with earlier findings of Wasim *et al* [14] and Kannahi and Vinotha [4]. The reason for good antimicrobial activity of *L.inermis* could be due to high concentration of antimicrobial compounds.Table2. Shows that most susceptible organism was *B.cereus* which was sensitive to all three plants extracts. *C.gigantea* extract exhibited antibacterial activity (7.4±0.25 mm to 17.3±0.21mm), *L inermis* extract showed antibacterial activity (9.2±0.13 mm to 20.3±0.19 mm) and *T.foenum-graecum* extract gave antibacterial activity (7.3±0.15 mm to 16.9±0.28 mm)of the different concentrations of plants extracts. It was of interesting that the extracts of *L. inermis*, *C.gigantea, T.foenum-graecum* exhibited antimicrobial activity against *S.aureus* MRSA (14.1±0.29 mm>13.1±0.25 mm >12.5±0.28 mm) respectively. Habbal *et al* [9] reported antimicrobial efficacy of *L. inermis* against *S.aureus* MRSA (16 mm).

Among the clinical isolates, Gram positive bacteria *B.cereus* showed the more sensitivity and among Gram negative, *E.coli* was most sensitive (Table 2).The inhibition zone against *B.cereus* and *E.coli* by the extracts of *C.gigantea* (7.2±0.21mm to 17.1±0.15 mm), *L.inermis* (8.4±0.18 mm to 19.1±0.29 mm)and *T.foenum-graecum* (6.6±0.19 mm to 16.1±0.19 mm) (Table 2). *L.inermis* showed highest antimicrobial activity against *E.coli* rather than *B.cereus.* This is in contrast to *Setzer et al* [15].

Antifungal activity of *C.gigantea* (9.3±0.28 mm to 21.7±0.21 mm), *L inermis* (9.3±0.28 mm to 21.7±0.21mm) and *T.foenum-graecum* (8.1±0.21 mm to 19.6±0.28 mm) of different extracts concentrations of plants (Table 3).*C.gigantea, L. inermis* and *T.foenum-graecum* demonstrated susceptibility against all the tested fungi [3, 16, 9]. *L.inermis* showed best antifungal activity against *A.flavus* [4].

Among the three medicinal plants extracts *L.inermis* showed best antimicrobial activity against tested bacteria and fungi. The MIC was determined of *L.inermis* extracts for all tested microorganism. The Gram positive bacteria *B.cereus* showed 4 mg/ml MIC (Table 4) and Gram negative bacteria *E.coli* showed 6mg/ml MIC, which is in contrast with the findings of *Setzer et al*[15].The MIC of fungi showed least for *A.flavus*(2 mg/ml) (Table 4), which is contrast with the findings of Wasim *et al*[14].All the three tested plant extracts showed dose dependent antimicrobial activity.

The TLC analysis of *L.inermis* revealed the presence of flavonoids and tannins. Bioautography results revealed the inhibition zones against the growth of *B.cereus* and *A.flavus* (Fig.1).The clear zones were located in two places, suggested that two compounds possessed the antimicrobial activity. The R_f values for inhibiting

compounds were 0.28 and 0.22 (Table 5).The compounds were identified by spraying sulfuric acid on plate C and 10 % $FeCl_3$ on plate D there by revealed the presence of flavonoids and tannins respectively (fig.1).

These findings corroborated with observations of Mattana *et al*[17] and Hatano *et al*[18]. Further investigations are needed for the purification and characterization of antimicrobial compounds which are detected in the TLC bioautography.

Table 2: The growth-inhibitory diameters (mm) of methanol extracts against the tested bacteria

Plant	Concentration of extract (mg/ml)	Test bacteria (zone of inhibition in mm)				
		S.aureus	***E. coli***	***MRSA***	***P.aeruginosa***	***B.cereus***
C.gigantea	0.125	4.8±0.12	7.2±0.21	3.8±0.14	6.1±0.21	7.4±0.25
	0.25	6.0±0.145	9.2±0.14	6.4±0.1	8.1±0.18	9.0±0.14
	0.5	9±0.11	12±0.1	8.1±0.15	10.6±0.25	11.5±0.25
	1.0	11.6±0.15	14.2±0.14	11.2±0.28	13.5±0.29	14.9±0.22
	2.0	14.2±0.17	17.1±0.15	13.1±0.25	15.2±0.25	17.3±0.21
L.inermis	0.125	6.3±0.17	8.4±0.18	5.6±0.18	7.8±0.26	9.2±0.13
	0.25	9.4±0.2	10.7±0.14	8.7±0.29	10.5±0.14	12.6±0.26
	0.5	12.8±0.15	13.3±0.21	11.4±0.14	13.2±0.19	14.3±0.19
	1.0	14.2±0.28	17.6±0.25	13.4±0.22	15.1±0.21	18.3±0.15
	2.0	16.5±0.15	19.1±0.29	14.1±0.29	17.2±0.18	20.3±0.19
T.foenum-graecum	0.125	3.8±0.18	6.6±0.19	3.1±0.23	5.4±0.21	7.3±0.15
	0.25	6±0.23	8.1±0.25	5.1±0.19	7.6±0.28	8.5±0.18
	0.5	7.3±0.17	11.2±0.18	6.9±0.21	10.6±0.26	11.5±0.21
	1.0	10.5±0.2	14.3±0.29	9.4±0.29	12.4±0.25	13.9±0.32
	2.0	13.2±0.18	16.1±0.19	12.5±0.28	14.6±0.21	16.9±0.28
Amoxicillin	(10µg/ml)	18.6±0.25	20.1±0.14	15.1±0.1	16.5±0.18	21.2±0.21

Mean ±SE (n=3)

Table 3: Antifungal activity of medicinal plants (mm) against the tested fungi

Plant	Concentration of extract (mg/ml)	Test fungi (zone of inhibition in mm)		
		C. albicans	***T.rubrum***	***A.flavus***
C.gigantea	0.125	8.2±0.22	6.3±0.15	9.3±0.28
	0.25	11.3±0.21	10.9±0.21	11.6±0.21
	0.5	14.6±0.4	13.9±0.36	15.3±0.34
	1.0	18.6±0.28	16.3±0.29	18.9±0.25
	2.0	21.1±0.32	19±0.29	21.7±0.21
L.inermis	0.125	9.4±0.28	8.6±0.25	10.3±0.15
	0.25	12.6±0.33	11.4±0.21	12.9±0.21
	0.5	14.9±0.21	14.6±0.28	16.5±0.29
	1.0	18.1±0.25	18±0.26	18.9±0.25
	2.0	20.6±0.25	20±0.216	21.3±0.24
T.foenum-graecum	0.125	7.4±0.18	5.5±0.24	8.1±0.21
	0.25	10.3±0.29	8.9±0.33	10.9±0.25
	0.5	11.4±0.21	11.1±0.25	13.2±0.26
	1.0	15.6±0.28	14.4±0.24	15.9±0.21
	2.0	19.1±0.21	17.3±0.21	19.6±0.28
Amphotericin B	(10 µg/ml)	22.1±0.36	21±0.2	23±0.4

Mean ±SE (n=3)

Table 4: Minimum Inhibitory Concentration (MIC) of *L.inermis*

Tested organism	MIC of *L.inermis* (mg/ml)
S. aureus	40
E. coli	6
MRSA	46
P. aeruginosa	12
B. cereus	4
C.albicans	4
T.rubrum	3
A.flavus	2

Table 5: Bioautographic R_f value of methanolic extracts of *L.inermis*

Name of compounds identified	Solvent system	R_f at which max. zone of inhibition occurred
Flavonoids	Toluene: ethyl acetate (1:1)	0.28
Tannins	Toluene: ethyl acetate (1:1)	0.22

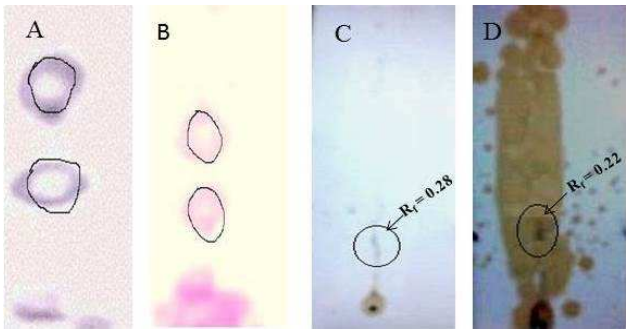

Fig. 1: Bioautography of methanolic extracts of *L.inermis* against *A.flavus* (A), *B.cereus* (B) and chromatogram for flavonoids (C), tannins (D).

The study concludes that the selected medicinal plants have a great potential antimicrobial activity against Gram positive, Gram negative and fungal species. Hence this study may help to identify, development of stable and active biological compounds for antimicrobial activity.

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