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Research Article

PRELIMINARY PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF *LAWSONIA INERMIS* LINN (HENNA) LEAF EXTRACTS AGAINST REFERENCE BACTERIAL STRAINS AND CLINICALLY IMPORTANT AMPC BETA-LACTAMASES PRODUCING *PROTEUS MIRABILIS*

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ABSTRACT

Phytochemical screening and antibacterial activity of aqueous, ethanol, methanol, ethyl acetate and chloroform extracts of *Lawsonia inermis* Linn leaves were tested against reference bacterial strains (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeuroginosa, Proteus mirabilis, Salmonella typhi, Vibrio cholerae, Staphylococcus aureus, Methicillin Resistant Staphylococcus aureus*) and clinical isolates (*Staphylococcus aureus* and AmpC β-lactamases producing *Proteus mirabilis*). The solvent extracts of *L. inermis* leaves exhibited profound antibacterial activity against the bacterial pathogens tested. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extracts for Gram positive species were between 34-64 µg and 39-74 µg . MIC and MBC of the extracts for Gram negative species were between 46-71 µg and 51-75 µg. The role of the plant material was apparent in inhibiting the growth of clinically important AmpC β-lactamases producing *Proteus mirabilis*, which have developed resistance to commonly prescribed antibiotics used for treating infections caused by uropathogens. The findings of the present study may emphasize the use of *L. inermis leaf* extracts with antibiotics for restricting the growth of clinically important multi-drug resistant uropathogens.

Keywords: *Lawsonia inermis*, AmpC β-lactamases, Antimicrobial activity, MIC, MBC

INTRODUCTION

Antibiotics are generally used against pathogenic bacteria. Currently, antibiotics are used to treat several human infectious diseases. Antibiotic are the chemical substances produced by one group of microorganisms that inhibits the growth of other pathogenic microbes [1]. The emergence of multi-drug resistant bacterial strains requires the use of alternative antimicrobials [2]. Bacterial pathogens are a serious problem to health and major cause of morbidity and mortality worldwide [3]. These microbes cause human diseases involving the skin and mucosal surfaces which constitute a serious illness particularly in tropical and subtropical developing countries[4]. Persistant search for new antimicrobial drugs is tremendously essential [5] due to the horizontal gene transfer of plasmids within the bacterial pathogens, which encode several enzymes, like ESBL's and AmpC beta-lactamase that hydrolyze the standard beta-lactum antibiotics.Many Plants are as used medicines in traditional medical system such as Ayurveda, Siddha and Unani besides folklore practices [6]. Plant extracts or plant-derived compounds are likely to provide a valuable source of new antimicrobial agents. Several plants have ability to treat the multiple drug resistance strains [7,8].

L. inermis Linn. *L. alba* (Henna) belongs to the family Lythraceae [9]. *L. inermis* is a glabrous splited plant or small tree (2 to 6 m in height). Leaves are small, opposite, entire edge egg-shaped to largely lanceolate, sub-sessile, about 1.5 to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, petiole short and glabrous acute or obtuse apex with narrowing base [10-12]. *L. inermis* is a well established aesthetic agent frequently used in colouring hair, skin and nails [13]. Henna also exhibits antimicrobial and anti dermatological properties. The plant also has antidiarrhoel, diuretic, emmanagogue and abortifacient prophetically and it is practically non-toxic [14]. The majority of phytochemical constituents of *L. inermis* are found to be possess significant anti inflammatory, analgesic and antipyretic activities [15]. Currently Henna is used as a counter stain in bacterial gram's staining [16]. The leaves are used as prophylactic in opposition to boils, burns, bruises, inflammations of skin and also against sore esophagus [17]. The roots of this plant are used for treating leprosy, strangury, and premature grey of hair and anti tuberculosis activity [18,19]. Other studies have reported that the alcoholic extracts of the leaves have high anti-inflammatory activity [20]. In the present study, qualitative phytochemical analysis and antibacterial activity with their minimal inhibitory concentration and minimal bactericidal concentration of *L. inermis* leaf extracts were analyzed against reference strains and clinical isolates of AmpC β-lactamases producing *P. mirabilis*.

MATERIALS AND METHODS

Plant Material

L. inermis leaves were collected from Cuddalore District, Tamil Nadu, India. The leaves were separated and rinsed with tap water, shade dried, homogenized to fine powder and stored in air tight container. For phytochemical analysis, 25 g of dried powder was extracted by successive extraction method using 200 ml of different solvents of increasing polarities. The solvents were evaporated by natural drying. The crude extracts were dissolved in 2% DMSO at a concentration of 25 mg/ml and extracts were stored at 4°C [21].

Qualitative Phytochemical analysis

Phytochemical analysis was carried out on the crude extracts of plant material using standard procedure to identify the following phytoconstituents; alkaloids, flavonids, saponin tannins, sterols, glycosides, phenol and resins [22,23].

Test organisms

Reference strains of both Gram positive and Gram negative bacteria were used for the study. The Gram positive bacteria were *S. aureus* ATCC 25923, *MRSA* ATCC 33592. The Gram negative bacteria consisted of *E. coli* ATCC 25922, *K. pneumoniae* MTCC 432, *P. aeuroginosa* ATCC 10145, *P. mirabilis* ATCC 7002 and *V. chloreae* MTCC 3904. One clinical isolate of *S. aureus* and five non repetitive AmpC β-lactamases producing *P. mirabilis* clinical isolates were used for the study. The reference strains were procured from American Type Culture Collection (ATCC) and Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The isolates were generous gift from Sri Ramachandra University, Porur, Chennai.

Antibiotic susceptibility testing

The standard Kirbuy-Bauer method was used for antimicrobial susceptibility testing. A sterile cotton swab was dipped into the bacterial culture and surplus removed by rotation of the swab against the side of the tube above the fluid level. The Mueller-Hinton agar medium (Hi-Media) was inoculated by streaking of the swab over the entire surface of the plate in 3 directions. The antibiotic

discs including Ampicillin (10 µg), Gentamicin (10 µg), Chloramphenicol (30 µg), Streptomycin (10 µg) and Penicillin-G (10 units) were placed on the plate and incubated at 37°C for 24 hours [25].

Antibacterial activity

The agar well diffusion method was performed for the determination of antimicrobial activity. The bacterial cultures were lawn cultured on Mueller -Hinton agar using sterile cotton swabs. The wells were cut on the agar plates using a cork borer (6 mm in diameter). 50 µl of the extracts (25 mg/ml) were pipetted into the well using a sterile micropipette. The plates were incubated at 37 °C for 24 hours. 2% DMSO used as negative control. The sensitivity to the antibiotics was interpreted after 24 hours incubation as per CLSI guidelines [MS100-S20, 2010] [26].

Determination of MIC and MBC

Different concentrations (10, 20, 30, 40, 50, 60, 70 and 80 µg/ml) of the leaf extracts were prepared in 10 ml of sterile Mueller-Hinton broth and the test organisms were inoculated and incubated at 37 °C for 24 hours. The culture tubes without extract served as a control. The MIC values were determined by monitoring the lowest concentration of the extract. The absence of turbidity indicates the inhibition of microbes. In the assay 40-70 µg/ml concentration characterize the inhibition of microbial growth. The optical density (OD) was measured at 600 nm for tests as well as control. Data were interpreted to calculate the MIC of crude extract against test organisms [27,28]. The MBC values of the extracts were determined by sub culturing 0.1ml from the last MIC test dilution that showed visible growth (turbidity) and all others in which there was no detectable growth on a fresh extract free solid medium and incubated at 37ºC for further 24 hours. The highest dilution that shows no single bacterial colony was considered as MBC [29].

RESULTS

Medicinal plants have a central role in the prophylactic and therapeutic strategies against human pathogens [19]. They are the backbone of traditional medicine system and the beneficial activity of plant extract on human welfare is due to the presence of bioactive compounds in the extract [20]. Some of these compounds may exert their activity by acting as antimicrobial agents. Almost all the antibacterial agents, isolated from the plants are aromatic or saturated organic compounds, they are most often obtained initially by ethanol or methanol extraction [22-24]. Qualitative phytochemical analysis of the ethanol, methanol, aqueous, chloroform and ethyl acetate extracts of *L.inermis* leaves were carried out to determine the presence of phytochemicals like tannins, flavonoids, saponins, steroids, alkaloids and glycosides (Table-1).

Table 1: Phytochemical screening of *L .inermis* using various extracts

Compounds	Plant extract				
	Aqueous	Ethanol	Methanol	Ethylacetate	chloroform
Alkaloids	-	+	-	+	+
Flavonids	-	-	-	-	-
Saponin	+	-	+	-	+
Tannins	-	+	+	-	-
Sterols	+	+	+	+	-
Glycosides	-	-	-	-	-
Phenol	+	+	-	-	+
Resins	-	-	-	-	-

+ = Positive, - = Negative

The results of the antibiotic susceptibility testing were interpreted by comparing with the standard antibiotic chart. All the reference strains and clinical isolates were resistant to beta-lactum antibiotic, namely Ampicillin and Penicillin-G. Streptomycin, Chloramphenicol and Gentamicin served as control and showed varying zones of inhibition against the reference strains and the clinical isolates tested (Table 2). Among Gram positive bacteria tested, maximum zone of inhibition was observed in ethanol, chloroform and ethyl acetate extracts against *S. aureus* (26 mm), *S. aureus* clinical isolate (26 mm) and *MRSA* (26 mm) respectively. Methanol extracts of *L. inermis* showed intermediate zones of inhibition against *S. aureus* (16 mm), *S. aureus* clinical isolate (17 mm) and *MRSA* (16 mm). Aqueous extracts of L.inermis exhibited moderate activity against the three *S. aureus* tested (Table 3). MIC of the extracts was between 34-64 µg. MBC was estimated to be in the range of 39-74 µg (Table 4). When considering Gram negative species, ethanol extracts of *L. inermis* had higher antibacterial activity against *P. mirabilis* (20 mm), *E.coli* (20 mm)„ *K. pneumoniae* (18 mm) and *P. aeuroginosa* (18 mm). Methanol extracts showed intermediate activity against all the organisms tested ranging from 13 mm to 15 mm (Table 3). Aqueous extracts of *L. inermis* showed moderate inhibition against the reference bacterial strains (11 mm to 15 mm). Ethyl acetate extracts showed maximum zone of inhibition against *P. mirabilis* (23 mm). No antibacterial activity was observed in chloroform extracts (Table 3). MIC of the extracts for Gram negative species were between 46-71 µg. MBC was estimated to be in the range of 51-75 µg (Table 4).

Table 2: Antibiotic susceptibility test against bacterial pathogens

Test organisms	Standard antibiotics (mm)				
	Ampicillin	Gentamycin	Chloramphenicol	Streptomycin	Penicillin-G
E.coli	R	R	35	16	R
K. pneumoniae	R	R	30	26	R
P. aeuroginosa	R	R	R	R	R
P. mirabilis	R	12	R	16	R
S. typhi	R	R	26	20	R
V. cholerae	R	10	32	26	R
S. aureus	R	24	28	19	R
MRSA	R	R	R	R	R
S. aureus (clinical isolate)	R	R	R	20	R
AmpC producing *P. mirabilis* clinical isolates					
Isolate 1	R	12	R	16	R
Isolate 2	R	11	R	10	R
Isolate 3	R	10	R	15	R
Isolate 4	R	14	R	14	R
Isolate 5	R	12	R	16	R

Table 3: Antibacterial activity of different extracts of *L. inermis* leaves against bacterial pathogens

Bacteria	Extract (25 mg/ml) Zone of inhibition(mm)				
	Aqueous	Ethanol	Methanol	Ethylacetate	Chloroform
E.coli	12	20	15	16	-
K. pneumoniae	12	18	14	18	-
P. aeuroginosa	11	18	14	18	-
P. mirabilis	15	20	15	23	-
S. typhi	14	17	15	-	-
S. aureus	17	20	16	26	-
MRSA	16	26	16	14	-
V. cholerae	12	14	13	-	-
S. aureus (Clinical isolate)	13	16	17	-	26
AmpC producing *P. mirabilis* clinical isolates					
Isolate 1	12	16	18	-	-
Isolate 2	10	12	16	-	-
Isolate 3	18	15	15	-	-
Isolate 4	14	16	17	-	-
Isolate 5	12	15	14	-	-

Table 4: MIC and MBC of different extracts of *L. inermis* leaves

Bacteria	Plant extract (µg)									
	Aqueous		Ethanol		Methanol		Ethyl acetate		Chloroform	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E.coli	63	68	46	52	54	59	54	59	-	-
K. pneumoniae	68	73	53	60	61	66	44	49	-	-
P. aeuroginosa	71	75	49	55	66	71	-	-	-	-
P. mirabilis	63	68	50	72	56	61	39	43	-	-
S. typhi	61	73	59	64	63	67	-	-	-	-
S. aureus	59	64	50	54	51	56	34	39	-	-
MRSA	54	59	49	54	59	64	69	73	-	-
V. cholerae	68	73	69	74	68	73	-	-	-	-
S. aureus (clinical isolate)	72	76	54	59	63	67	-	-	69	74
AmpC producing *P. mirabilis* clinical isolates										
Isolate 1	63	68	61	66	48	55	-	-	-	-
Isolate 2	69	74	62	67	55	57	-	-	-	-
Isolate 3	72	77	63	69	62	67	-	-	-	-
isolate 4	61	66	54	59	59	-	-	-	-	-
isolate 5	63	68	63	68	61	-	-	-	-	-

MIC - minimal inhibitory concentration; MBC - minimal bactericidal concentration

DISCUSSION

L. inermis (Henna) exhibits antimicrobial, antidiarrhoel and anti-dermatological properties[14]. The leaves of *L. inermis* are nono-toxic and are used to cure boils, burns, bruises and other skin infections[17]. The present study apparently confirms the antibacterial activity of aqueous, ethanol, methanol, ethyl acetate and chloroform extracts of *L. inermis* leaves. Previous studies reported the antimicrobial activity of *L. inermis* leaf extracts, but, our study established the biological benefits in leaves of *L. inermis* against bacterial pathogens and AmpC producing clinical isolates of *P. mirabilis*. The antimicrobial activities of plant extracts depend on a variety of factors; the environmental and climatic conditions under which the plant dwells, the solvent that is used for the extraction, the protocol and test concentrations [3,][22,23][]. In the present study, the plant materials were extracted with different organic solvents in increasing polarity order. Such sequential extraction yields the active compounds from plant material based on their polarity, and also reduce the antagonistic effect of compounds in the extract. Better antibacterial effect was eminent in *L. inermis* leaves subjected to successive extraction against reference strains and clinical isolates. The extracts of *L. inermis* leaves revealed profound antibacterial activity against clinically important AmpC β-lactamases producing *P. mirabilis*, which showed remarkable resistance towards third generation cephamycins and cephalosporins. AmpC beta-lactamases are clinically significant cephalosporinases, which confer resistance to cephamycins, penicillins, and β -lactam- β-lactamase inhibitor combinations. Certain microbes acquire these enzymes by horizontal gene transfer of the plasmid DNA. Continual treatment with standard antibiotics lead to the genesis of these enzymes worldwide in diverse species of *Enterobacteriaceae,* such as *P. mirabilis, K. pneumoniae*, *K. oxytoca*, *E. coli* and *Salmonella* spp [30]. Our results recommend the use of henna extracts against AmpC β-lactamases producing clinically important *P. mirabilis* due to its enhanced antibacterial activity.

CONCLUSION

The findings of the present work revealed the approaching use of *L. inermis* for treatment of AmpC β-lactamases producing clinical isolates. Future study is necessary to identify the plant compounds responsible for the bacterial growth arrest.

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