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Research Article

PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL POTENTIALS OF *ALANGIUM SALVIFOLIUM* AND *PIPER LONGUM* AGAINST MULTI-DRUG RESISTANT BACTERIA FROM CLINICAL ISOLATES

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ABSTRACT

The antimicrobial activities of various solvent extracts of *Alangium salviifolium* and *Piper longum* were evaluated against clinically proved multi-drug resistant bacteria (Methicillin-resistant *Staphylococcus aureus*, *Enterococcus* sp., *Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp. and reference strains of bacteria (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) by using agar well diffusion assay. The patterns of inhibition varied with the plant extract, the solvent used for extraction, and the organism tested. Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus* sp. were the most inhibited microorganisms. The highest antimicrobial potentials were observed for the hexane and acetone extracts of *A. salviifolium* and *P. longum*, displaying maximum inhibitory zone of 18 mm against *Enterococcus* sp. *Acinetobacter* sp. was susceptible only to hexane extract of *A. salviifolium*. However, aqueous extracts did not present any antibacterial activity. Phytochemical screening showed that all the extracts contain alkaloids and reducing sugars while anthraquinones were absent in all the extracts tested. Hexane and acetone extracts were separated using TLC and relative mobilities of bioactive components showing significant inhibitory zones against *S. aureus* MRSA and *Enterococcus* sp. were determined by bioautography agar overlay assay.

Keywords: Agar well diffusion, Multi-drug resistant, Phytochemical analysis, TLC bioautography

INTRODUCTION

In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by bacteria and other microbes. Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the Twentieth Century. However, disease-causing microbes that have become resistant to antibiotic drug therapy are an increasing public health problem [1]. It is estimated that about 70 per cent of the bacteria that cause infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Looking at this scenario 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.

Medicinal plants have a diverse array of natural phytochemicals that have complementary and overlapping actions, including antioxidant effects, modulation of detoxification enzymes, stimulation of immune system, reduction of inflammation, modulation of steroid metabolism and antibacterial and antiviral effects [2]. In support most medicinal plants have antimicrobial properties and their use is greatest in tropics where the diversity and growth rates of microorganisms are highest. *Alangium salviifolium* Linn (Alangiaceae) is a small deciduous tree or shrub, which grows in the wild throughout the hotter parts of India. *A. salviifolium* has various medicinal properties and used as laxative, astringent, pungent, purgative, alleviates spasms, anthelmintic, emetic, antiprotozoa, hypoglycemic, antibacterial [3, 4]. *Piper longum* is a native of the Indo-Malaya region. It is reported to possess antiasthmatic, hepatoprotective, hypocholesterolaemic, anti-inflammatory, antiamoebic and antibacterial activities [5]. The present study aimed at evaluating the *in vitro* antibacterial activity of various extracts of *Alangium salvifolium* and *Piper longum* against multi-drug resistant (MDR) Gram-positive (Methicillin-resistant *Staphylococcus aureus* and *Enterococcus* sp.) and Gram-negative (*Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp.) bacterial strains isolated from human infections.

MATERIALS AND METHODS

Plant material and extracts preparation

The seeds of *A. salviifolium* and roots of *P. longum* was oven dried at 50ºC, ground and extracted successively with n-hexane, acetone, methanol, ethanol and water. The collected plant materials were identified and authenticated by Prof. P. D. Sharma, Retd. Botanist, Delhi University, Delhi, India. The different extracts were concentrated at reduced pressure to dryness using a soxhlet evaporator for 48 h [6]. After complete solvent evaporation, extracts were dissolved in 10% DMSO to a final concentration of 20 mg/ml and stored at 5°C in labeled sterile screw-capped bottles until assayed.

Bacterial strains

The pure cultures of the bacteria with their antibiotic resistance profiles were obtained from the Department of Microbiology, Rajiv Gandhi Cancer Research Institute, Delhi, India (Table: 1). These include: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus* sp., *Klebsiella* sp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter* sp. All were collected in slants containing Mueller-Hinton agar and few colonies from these cultures were inoculated into Mueller-Hinton broth, incubated at 37^{0}C for 24 h before use. Standard strains *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used for quality control.

Phytochemical analysis

The extracts were subjected to phytochemical screening for the presence of saponins, tannins, steroids, phlobatanins, anthraquinones, cardiac glycosides, alkaloids, reducing sugars and flavonoids by using wet reactions following the procedures described by Sofowora [7] and Trease and Evans [8].

Antibacterial activity assay

The agar well diffusion method [9] was employed with slight modifications to determine the antibacterial activities for various solvent and aqueous extracts of *A. salvifolium* and *P. longum*. About 25 ml of Mueller-Hinton agar was poured into each petri plate. Once the agar solidified, the bacteria were inoculated on the surface of the plates (1 × 10^8 cfu/ml). Subsequently, the surface of the agar was punched with a 6 mm diameter wells. Each well was filled with 50 µl of each plant extract. The concentration of the extracts employed was 20 mg/ml. The reference antibiotic discs of Imepenem (10 µg) and Vancomycin (30 µg) were used as the positive controls. Control wells containing the same volume of hexane, acetone, methanol, distilled water and DMSO were made. After 24 h incubation at 37^{0}C, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition.

TLC bioautography assay

Hexane and acetone extracts showing significant antimicrobial activity against *S. aureus* MRSA and *Enterococcus* sp. were investigated by thin layer chromatography (TLC) bioautographic agar-overlay method [10]. About 10 µl of each extract was chromatographed on pre-coated aluminium silica gel G 25 plates with toluene: ethyl acetate (93:7) as mobile phase. The developed TLC plates were dried and thinly overlaid with molten Mueller-Hinton agar inoculated with an overnight culture of the test bacteria. The plates were incubated in dark and humid chamber at 37^{0}C for 24 h. After incubation plates were sprayed with aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride and further incubated at 37^{0}C for 4 h. Microbial growth inhibition appeared as clear zones around active compounds against a pink background. The plates were in duplicate. One set was used for bioautography experiment (plate B) and the other was intended for the reference chromatogram (plate A). The reference plate was observed in UV light to see if the separated spots were UV active after which it was sprayed with vanillin sulphuric acid (2%) spray reagent. After spraying, the chromatogram was heated at 110°C in an incubator to allow for optimal colour development. The *Rf* of the inhibition zones on plate B was compared with the *Rf* of reference chromatogram (plate A).The experiments were repeated twice.

Table 1: Antibiotic resistance profile of various Gram-positive and Gram-negative bacterial isolates used

				Test Bacteria			
Antibiotics	***Ksp*1**	***Ksp*2**	***Ec*1**	***Pa***	***Asp***	***Esp***	***Sa* MRSA**	***Sa* 1**
AK	S	R	S	S	R	R	S	R
AC	R	R	R	R	R	R	R	R
CFX	R	R	R	R	R	R	R	R
CS	R	R	S	S	S	R	S	R
CE	R	R	R	R	R	R	R	R
CI	R	R	R	R	R	R	R	R
CF	R	R	R	S	R	R	R	R
GF	S	R	S	S	R	R	S	R
G	S	R	R	S	R	R	R	R
I	S	S	S	S	S	R	S	R
LE	S	R	R	S	R	R	S	R
MR	S	R	S	S	R	R	R	R
OF	R	R	R	S	R	R	R	R
PT	S	R	S	S	S	R	R	R
VA	-	-	-	-	-	S	S	S
LZ	-	-	-	-	-	S	S	S

a) AK: Amikacin, b) AC: Amoxycillin/Clavulanic acid, c) CFX: Cefixime, d) CS: Cefoperazone +Sulbactum, e) CE: Cefotaxime, f) CI: Ceftriaxone, g) CF: Ciprofloxacin, h) GF: Gatifloxacin, i) G: Gentamicin, j) I: Imipenem, k) LE: Levofloxacin, l) MR: Meropenem, m) OF: Ofloxacin, n) PT: Piperacillin/Tazobactam, o) VA: Vancomycin, p) LZ: Linezolid, q) R: Resistant, r) S: Sensitive, s) *Ksp*: *Klebsiella* sp., t) *Ec*: *Escherichia coli,* u) *Pa*: *Pseudomonas aeruginosa*, v) *Asp*: *Acinetobacter* sp., w) *Esp*: *Enterococcus* sp. x) *Sa*: *Staphylococcus aureus*.

RESULTS AND DISCUSSION

Phytochemical analysis

Phytochemical screening of the various extracts revealed that reducing sugars, saponins, cardiac glycosides and alkaloids are generally present in all the extracts. However, some phytoconstituents were absent in some extracts (Table: 2). Anthraquinones were absent in all the extracts. In a similar study, alkaloids, flavonoids, phenols, steroids, glycosides, tannins, oils and fats, saponins and fatty acids were found present in the fruit and seed extracts of *A. salvifolium* [11]. The chemistry of *Piper* species has been widely investigated that have led to the isolation of a number of physiologically active compounds such as alkaloids/amides, propenylphenols, lignans, neolignans, terpenes, steroids, kawapyrones, piperolides, chalcones, di-hydrochalcones, flavones and flavanones [12].

Table 2: Phytochemical analysis of various extracts of *Alangium salvifolium* and *Piper longum*

	Alangium salvifolium				***Piper longum***			
Phytoconstituents	**H**	**A**	**M**	**E**	**H**	**A**	**M**	**E**
Saponins	-	+	+	+	+	-	++	+
Flavonoids	-	+	+	+	-	+	-	-
Steroids	++	+	-	-	++	++	+	+
Phlobatanins	-	+	-	-	-	-	-	-
Tannins	-	++	++	++	-	-	-	-
Alkaloids	+	++	++	+	++	++	++	+
Cardiac Glycosides	++	-	+	+	++	++	+	-
Reducing Sugars	+	+	+	++	+	+	+	+
Anthraquinones	-	-	-	-	-	-	-	-

H: hexane extract, A: acetone extract, M: methanol extract, E: ethanol extract, +: moderate amount, ++: higher amount, -: not present.

Antibacterial activity assay

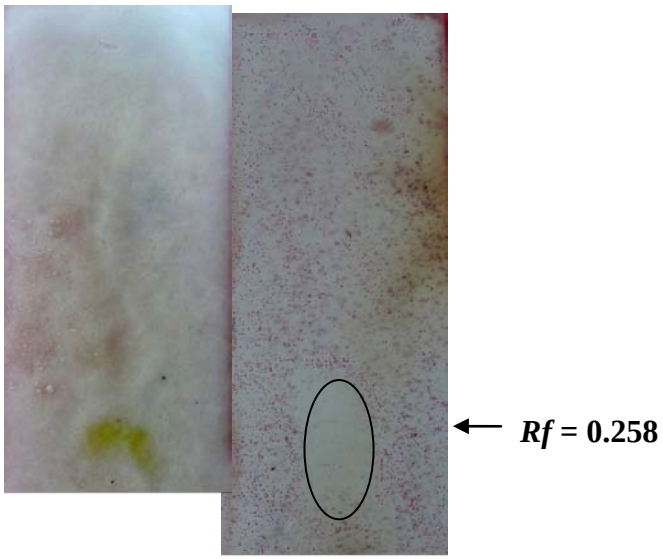
The antibacterial activity of *A. salvifolium* and *P. longum* extracts on the agar plates varied for the different solvents. The hexane extract from *P. longum* presented the highest activity against both *S. aureus* ATCC 25923 and *S. aureus* MRSA. MDR isolates *S. aureus* MRSA, *E. coli*, *Klebsiella* sp. 1 and *Enterococcus* sp. were found to be susceptible to three of the four solvent extracts of both *A. salvifolium* and *P. longum* (Table: 3). The growth of *Acinetobacter* sp. was only inhibited by the hexane extract of *A. salvifolium* with inhibition zone of 15 mm. However, *P. aeruginosa* was inhibited by both methanol and ethanol extracts of *P. longum*. Jain *et al.* [4] demonstrated that extracts of *A. salvifolium* had activity against several bacteria, especially *S. aureus* and *E. coli*, inhibiting the growth of these bacteria with minimum inhibitory concentration ranging between 0.130 to 0.520 mg/ml. In another study, ethanol, methanol and acetone fruit extracts of *P. longum* were found to show moderate activity against all the tested bacteria [13]. According to Joy *et al.* [14], hot ethyl acetate extract compared to the cold hexane: water (1:1) extract showed more potency against *E coli* than *Bacillus subtilis* and was found to be less active for *S. aureus*. Moreover, none of the extracts were active against *S. aureus* 1 and *Klebsiella* sp. 2 (Table:3). Water extracts exhibited no inhibitory effect against the assayed bacteria.

This observation confirmed the evidence from a previous study which reported that alcohol is a better solvent for extraction of antimicrobial substances from medicinal plants than water [15]. The control plate representing the various solvents did not exhibit inhibition on the tested bacteria where as standard antibiotics Imepenem and Vancomycin produced significantly larger inhibition zones against Gram-negative and Gram-positive bacteria respectively.

Table 3: Antibacterial activity of *Alangium salvifolium* and *Piper longum* by agar well diffusion assay

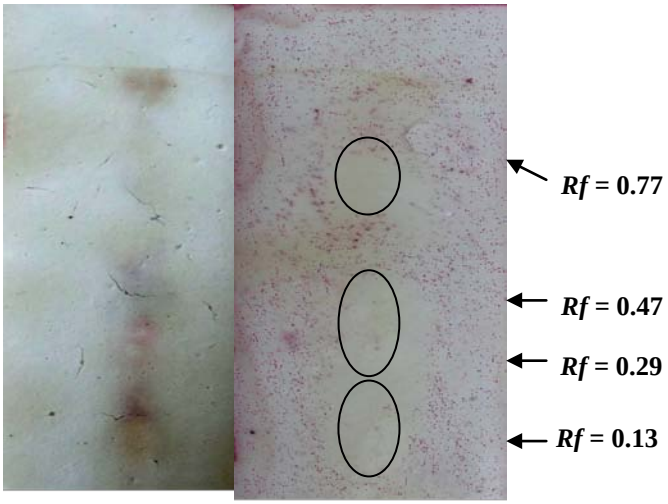
	Zone of inhibition (in mm)							
	Alangium salvifolium				*Piper longum*			
Test Bacteria	**H**	**A**	**M**	**E**	**H**	**A**	**M**	**E**
S. aureus ATCC*	18	15	14	11	27	18	22	-
S. aureus MRSA	17	16	12	-	18	15	12	-
S. aureus 1	-	-	-	-	-	-	-	-
Klebsiella sp. 1	14	14	-	8	11	13	-	7
Klebsiella sp. 2	-	-	-	-	-	-	-	-
E. coli ATCC#	15	18	16	12	10	17	14	12
E. coli	-	15	16	10	-	12	13	12
Enterococcus sp.	18	17	17	-	16	18	13	-
Acinetobacter sp.	15	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	10	13

Zone of inhibition is the mean of three readings, H: hexane extract, A: acetone extract, M: methanol extract, E: ethanol extract (20 mg/ml), -: no inhibition, *: *S. aureus* ATCC 25923, #: *E. coli* ATCC 25922.



(Plate A) **(Plate B)**

Fig. 1: Thin layer chromatography plates were run in duplicates and one set was visualized after spraying with vanillin sulphuric acid (plate A), the other set was used for bioautography with (plate B) as described in the methodology. Arrow indicates antibacterial activity of hexane extract of *Alangium salfifolium* against *Enterocoocus* sp. at *Rf* value 0.258.



(Plate A) **(Plate B)**

Fig. 2: Thin layer chromatography plates were run in duplicates and one set was visualized after spraying with vanillin sulphuric acid (plate A), the other set was used for bioautography (plate B) as described in the methodology. Arrow indicates antibacterial activity of hexane extract of *Piper longum* against *S. aureus* MRSA.

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