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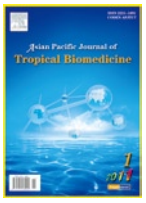

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Evaluation of antimicrobial properties of four plant extracts against human pathogens

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ABSTRACT

Objective: To investigate the antibacterial activity of the extracts of *Alternanthera philoxeroides* (*A. philoxeroides*), *Plumeria obtusa* (*P. obtusa*), *Polyalthia cerasoides* (*P. cerasoides*) and *Ixora acuminate* (*I. acuminate*) against human pathogens. **Methods:** Aqueous and chloroform: methanol (1:1) extracts of the dried leaf of *A. philoxeroides*, flowers of *P. obtusa*, fruits of *P. cerasoides* and flowers of *I. acuminate* were tested *in vitro* by the disk diffusion method against four bacterial strains, namely, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Susceptibility of four reference bacterial strains to some antibiotics in nutrient agar was also tested. Minimal inhibitory concentration (MIC) values were determined and qualitative phytochemical analysis of the crude extract of the tested plant parts was done. **Results:** Both the aqueous and the chloroform: methanol (1:1) extracts of *P. cerasoides* showed the strongest activity, followed by flowers of *P. obtusa*, leaves of *A. philoxeroides* and flowers of *I. acuminate*. Aqueous extracts of all the plant parts appeared to have less antibacterial activity than the chloroform: methanol (1:1) extracts. The result of phytochemical analysis of the crude extract of the tested plants showed that flavonoid was absent from all plant parts whereas steroid was present in all tested plant parts. **Conclusions:** The results support that these plant extracts can be used for the treatment of bacterial diseases.

1. Introduction

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are sources of many potent and powerful drugs[1]. Over the years, World Health Organization[2] has advocated traditional medicines as safe remedies for ailments of both microbial and non-microbial origins. Several herbs were known to possess medicinal values including antimicrobial properties[3]. The extensive use of synthetic drugs, excessive unwanted medication will cause increasing side effects in the body, sometimes, the toxic effects produced by the administration of drugs are much more a serious problem than that of the disease itself. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to common people. The rising incidence in multidrug resistance amongst pathogenic microbes has further necessitated the need to search for newer antibiotic sources. Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trails[4,5]. The selection of crude plant extracts for screening programs has the potential of being more successful in its initial steps than the screening of pure compounds that are isolated from natural products[6].

The present study was designed to determine the role of aqueous and chloroform: methanol (1:1) extracts of *Alternanthera philoxeroides* (*A. philoxeroides*), *Plumeria obtusa* (*P. obtusa*), *Polyalthia cerasoides* (*P. cerasoides*) and *Ixora acuminate* (*I. acuminate*) for potential antibacterial activity against human pathogenic two gram-positive bacteria *i.e. Staphylococcus aureus* (*S. aureus*) and *Bacillus subtilis* (*B. subtilis*) and two gram-negative bacteria *i.e. Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). The observed inhibition zones were measured

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in mm. The study also extended up to the phytochemical analysis of the plant extracts to have an idea about the bioactive principles responsible for antimicrobial properties.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study consisted of mature leaves of *A. philoxeroides*, flowers of *P. obtusa*, fruits of *P. cerasoides* and flowers of *I. acuminata* collected from the outskirts of Burdwan (23 ° 16′N, 87 ° 54′E), West Bengal, India, during the spring season (mid March to mid April 2010). Plant materials were initially rinsed with distilled water and dried on paper towels in the laboratory at (37±1) ℃ for 24 h.

2.2. Test microorganisms

Four bacterial strains were used for the study. Gram-positive bacteria include *S. aureus* MTCC 2940 and *B. subtilis* MTCC 441, and gram-negative bacteria include *E. coli* MTCC 739 and *P. aeruginosa* MTCC 2453. All the tested strains are reference strains and were collected from the Microbiology Laboratory of Burdwan Medical College. The bacterial cultures were maintained in nutrient broth (Himedia, M002) at 37 ℃ and maintained on nutrient agar (Himedia, MM012) slants at 4 ℃.

2.3. Preparation and preservation of plant extract

2.3.1. Preparation of aqueous extract

Each of the four samples was weighed out (50 g) and soaked separately in 200 mL of cold water contained in conical flasks stoppered with rubber corks and left undisturbed for 24 h. They were then filtered off using sterile filter papers (Whattman No. 1) into clean conical flasks and subjected to water bath evaporation, where the aqueous solvents were evaporated at boiling temperature of 100 ℃. The standard extracts thus obtained were then stored at 4 ℃ in a refrigerator until further use[7].

2.3.2. Preparation of chloroform: methanol (1:1) extract

After drying, the plant materials were ground separately in a grinding machine (MX-110 PN, Japan) in the laboratory. Exposure to sunlight was avoided to prevent the loss of active components. The chloroform: methanol (1:1) extraction fluid (500 mL) was mixed with each of powdered plant materials (50 g). The mixtures were then kept for 21 days in tightly sealed vessels at room temperature, protected from sunlight, and stirred thoroughly several times a day with sterile glass rods. The mixtures thus obtained were filtered through Whatman No. 1 filter papers. The extracted liquids were subjected to rotary evaporation in order to remove the chloroform: methanol (1:1). The semisolid extracts produced were kept at 80 ℃ (REVCO model No. ULT 790-3-V 32) in a freezer overnight and then subjected to freeze-drying for 24 h at 60 ℃ in a 200 mL vacuum. Then the extracts were stored in an airtight container at 4 ℃ in the refrigerator until further use. All the dried extracts were exposed to UV rays (200-400 nm) for 24 h and checked frequently for sterility by streaking on nutrient agar plates[8].

2.4. Antibacterial assay

Antibiogram was done by disc diffusion method[9,10] using plant extracts and commonly used antibiotics. The test quantity of specific extracts were dissolved in either distilled water or dimethylsulphoxide (DMSO), depending upon the solubility of the extracts. The dissolution of the organic extracts [chloroform: methanol (1:1)] were aided by 1% (v/v) DMSO and that of the aqueous extract was aided by water, which did not affect the growth of microorganisms, in accordance with our control experiments. The surfaces of media were inoculated with bacteria from a broth culture. High-potency bio-discs (Himedia) were placed on the agar. After 18 h of incubation at a specific temperature [(30 ±1) ℃ for *B. subtilis* and 37 ℃ for *S. aureus*, *E. coli*, and *P. aeruginosa*], the plates were examined and the diameters of the inhibition zones were measured to the nearest millimeter.

2.5. Phytochemical analysis of the plant extracts

Phytochemical analysis of all the aqueous plant extracts was carried out by suitable methodologies in search of active ingredient responsible for bacterial toxicity. The phytochemicals included under study were saponins, terpenoids, alkaloid, steroids, tannin, flavonoids, cardiac glycosides and free glycoside bound anthraquinones and the analysis was carried out according to the methodologies of Edeoga *et al*[11].

2.6. Statistical analysis

Since the readings of control (distilled water) experiments in the *in vitro* antibacterial studies of those plants were zero, the data were analyzed by simple arithmetic means of the different extracts, and the standard errors were compared with the control.

3. Results

All the plants assayed in this study are commonly used as medicinal plants in different areas of India and other parts of the world. Their medicinal properties were presented in Table 1.

Table 1
Description of plants.

Plants	Common name	Systemic position	Botanical description	Properties
A. philoxeroides	Alligatorweed	Caryophyllales: Amaranthaceae	It is an immersed aquatic plant. Stems are long, branched, and hollow. Leaves are simple, elliptic, and have smooth margins. Flowers are papery ball-shaped.	Its twigs and leaves are a snakebite antidote[17].
P. obtusa	White Champa	Gentianales: Apocynaceae	It is a large evergreen shrub with narrow elongated leaves, large highly perfumed white flowers with a yellow center.	The milky sap of the stem and leaf is applied to skin diseases such as herpes, and ulcers. Its bark is used as plaster over hard tumors, the seeds while the latex is used as purgative, cardiotonic, diuretic and hypotensive[18].
P. cerasoides	Devadaru	Magnoliales: Annonaceae	It is a deciduous tree up to 10 m tall. Leaves are simple, alternate and distichous, lanceolate, apex acute. Flowers are arranged in a one or few flowered inflorescence. Fruit lets up to 0.6 cm in diameter, in clusters of one seeded berry-like fruits.	Wood is sometimes used for veneer and plywood, ripe fruitletsh edible[19].
I. acuminata	Rangan	Gentianales: Rubiaceae	Plants possess leathery leaves, ranging from 3 to 6 inches in length, and produce large clusters of tiny flowers in the summer.	Red *Ixora* flowers are commonly used in Indian folk medicine[20].

Table 2
Antibacterial activity of specific concentration of aqueous and chloroform: methanol (1:1) extracts of medicinal plant compared with control by disc diffusion method.

Plants	Extraction solvent (35 mg/disc)	Diameter of the inhibitory zones (mm)			
		S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*
A. philoxeroides (leaf)	Aqueous	13.33±0.18	13.60±0.35	14.20±0.12	17.13±0.09
	Chloroform: methanol (1:1)	16.20±0.12	18.27±0.15	14.80±0.15	19.23±0.15
P. obtusa (flower)	Aqueous	14.43±0.34	15.07±0.12	16.40±0.23	21.33±0.15
	Chloroform: methanol (1:1)	15.10±0.05	18.23±0.15	20.37±0.27	22.27±0.22
P. cerasoides (fruit)	Aqueous	14.03±0.08	15.20±0.15	18.57±0.32	18.37±0.15
	Chloroform: methanol (1:1)	30.80±0.57	26.30±0.25	28.27±0.32	30.33±0.24
I. acuminate (flower)	Aqueous	12.53±0.27	14.23±0.19	16.10±0.20	15.23±0.19
	Chloroform: methanol (1:1)	14.50±0.27	15.37±0.27	18.00±0.05	16.37±0.27
Distilled water	_	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
DMSO	_	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Table 3
Susceptibility of four reference bacterial strains to some antibiotics in nutrient agar.

Antibiotics (μg/mL)	Diameter of the inhibitory zones (mm)			
	P. aeruginosa	*S. aureus*	*E. coli*	*B. subtilis*
Amoxycillin (30)	14	0	7	0
Ampicillin (10)	0	0	0	0
Amikacin (30)	28	28	0	29
Cotrimoxazole (25)	38	21	0	14
Ciprofloxacin (5)	32	25	27	31
Chloramphenicol (30)	30	25	0	22
Cloxacillin (1)	0	0	0	0
Cefadroxil (30)	28	0	0	0
Cefuroxime (30)	0	0	0	0
Doxycycline (30)	28	8	20	30
Erythromycin (15)	25	12	0	5
Gentamycin (10)	21	23	22	19
Gatifloxacin (10)	39	29	37	37
Kanamycin (30	33	11	10	26
Lomefloxacin (10)	29	22	25	34
Levofloxacin (5)	32	26	23	30
Nalidixic acid (30)	0	0	11	20
Norfloxacin (10)	25	17	24	0
Ofloxacin (5)	27	18	20	20
Penicillin-G (10)	0	0	0	0
Sparfloxacin (10)	34	26	30	36
Tobramycin (10)	22	16	0	17
Tetracyclin (30)	0	0	12	25

Table 4
MIC of different extracts by dilution method.

Test material	Bacteria	MIC values (μg/mL)	
		Aqueous extract	Methanol extract
A. philoxeroides	*P. aeruginosa*	76.25	40.25
	S. aureus	75.50	35.25
	E. coli	80.00	45.00
	B. subtilis	76.25	45.25
P. obtusa	*P. aeruginosa*	77.25	46.25
	S. aureus	70.50	51.50
	E. coli	245.00	97.25
	B. subtilis	90.50	80.25
P. cerasoides	*P. aeruginosa*	250.00	55.00
	S. aureus	87.50	77.50
	E. coli	90.00	46.00
	B. subtilis	79.50	46.25
I. acuminata	*P. aeruginosa*	68.50	57.50
	S. aureus	78.25	56.25
	E. coli	57.00	47.50
	B. subtilis	145.00	74.25

The antibacterial activities of the mature leaves of A. philoxeroides, flowers of P. obtusa, fruits of P. cerasoides and flowers of I. acuminate in different solvents like aqueous and chloroform: methanol (1:1) against *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus* were shown in Table 2, respectively. Antibiogram of the commonly used antibiotics was shown in Table 3. The MIC values of the tested plant extracts against the tested microorganisms were shown in Table 4. The results of preliminary qualitative phytochemical analysis of all the plants were presented in Table 5.

Table 5
Result of qualitative phytochemical analysis of the crude extract of the tested plants.

Plants	Parts	Result of phytochemical analysis							
		Tannin	Saponin	Steroid	Flavonoid	Terpenoid	Cardiac glycosides	Alkaloid	Free glycoside bound anthraquinones
A. philoxeroides	Leaf	−−	++	++	−−	−−	−−	++	++
P. obtusa	Flower	−−	−−	++	−−	++	++	++	++
P. cerasoides	fruit	−−	++	++	−−	++	−−	−−	−−
I. acuminata	flower	++	++	++	−−	++	−−	−−	−−

++: presence; −−: absence.

4. Discussion

The results indicate that the aqueous extracts of all the plants studied showed antibacterial activities toward the gram-positive bacteria (*S. aureus* and *B. subtilis*) as well as gram-negative bacteria (*E. coli* and *P. aeruginosa*). The chloroform: methanol (1:1) solvent extracts of all the plant parts showed more effective result than those of aqueous extracts against all bacterial strains. Thus it was evident that the organic extracts were more effective than the aqueous extracts. It was seen that both aqueous and chloroform: methanol (1:1) solvent extracts of all four plant parts showed better result in gram-negative bacteria than those of gram-positive bacteria. The highest antimicrobial activity was recorded in fruits of *P. cerasoides* followed by flowers of *P. obtuse*, leaves of *A. philoxeroides* and flowers of *I. acuminate*. The result of antibiogram which is tested against all four bacterial strains showed that all four bacterial strains were resistant to ampicillin, cloxacillin, cefuroxime and penicillin-G. The phytochemical analysis of the plant extracts reveals the presence of several bioactive secondary metabolites that singly or in combinations may be responsible for the antimicrobial activity. From the result it was also observed that steroid was present in all four plant parts, whereas flavonoid was absent in all four plant material. Tanin and cardiac glycosides were least common and saponins and terpenoids were present in three plant parts. Aqueous extract of *P. obtusa* showed the best result against *P. aeruginosa* having MIC value 77.25 μg/mL in aqueous extract. Fruits of *P. cerasoides* showed the

best result against *S. aureus* having MIC value 77.50 μg/mL in chloroform: methanol (1:1) extract. The demonstration of antimicrobial activity against both gram-positive and gram-negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds as also reported by Masola *et al*[12]. It had been shown that when solvents like ethanol, hexane and methanol are used to extract plants, most of them are able to exhibit inhibitory effect on both gram positive and gram negative bacteria[13,16]. Phytochemical constituents such as tannins, saponins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that serve as defense mechanisms against predation by many microorganisms, insects and other herbivores[17-23].

In conclusion, it is suggested that these plants may be recommended as useful sources to prepare natural bioactive products from which we can develop new antimicrobial drugs which will be cost-effective because the plants are freely available. In the search for new pharmaceuticals, screening of such various natural organic compounds and identification of active agents must be considered as a fruitful approach.

Conflict of interest statement

We declare that we have no conflict of interest.

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