

Research Article

Evaluation of the Antimicrobial Activity of Some Medicinal Plants against Enteric Bacteria with Particular Reference to Multi-Drug Resistant *Vibrio cholerae*

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Abstract

Purpose: To evaluate the anti-enterobacterial potential of nine ethnobotanically selected plants traditionally used in different parts of India for the treatment of gastrointestinal disorders such as cholera, diarrhea or dysentery.

Methods: The methanol extracts of these plants were screened for antibacterial activity against 11 strains of enteropathogenic bacteria, including multi-drug resistant *Vibrio cholerae* (serotypes O1, O139, and non-O1, non-O139), using broth microdilution method. Ampicillin was used as a positive reference standard to determine the sensitivity of the strains. Phytochemical screening was carried out for phenolics and flavonoids.

Results: All of these plants had bactericidal activity against at least one of the test microorganisms with minimum inhibitory concentration (MIC) ranging from 0.125 to 32 mg/ml and minimum bactericidal concentration (MBC) ranging from 0.25 to 32 mg/ml. Among these, *Terminalia chebula* Retz. (Combretaceae) and *Syzygium cumini* (L.) Skeels (Myrtaceae) showed the most promising broad spectrum antibacterial properties, inhibiting all of the strains tested, especially *Vibrio cholerae*, *Aeromonas hydrophila* and *Bacillus subtilis*, with MBC ranging from 0.25 to 4 mg/ml. Phenolics and flavonoids were found to be present in the extracts.

Conclusion: The findings provide support for the use of this plant in traditional medicine for treatment of diarrhea, especially cholera.

Keywords: Antimicrobial activity; Cholera; Diarrhea; Indian medicinal plants; Enteric bacteria; Indigenous

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INTRODUCTION

Morbidity and mortality due to diarrhoea continues to be a major problem in many developing countries, including India and Bangladesh, especially amongst children. Infections due to a variety of bacterial etiologic agents, such as pathogenic *Escherichia coli*, *Vibrio cholerae*, *Aeromonas spp.*, *Shigella spp.*, *Salmonella spp.*, *Pseudomonas spp.*, *Klebsiella spp.*, *Campylobacter spp.*, and *Staphylococcus aureus* are most common. However, antibiotic resistance is a major clinical problem in treating infections caused by these microorganisms. Many readily available plants in India are used in traditional folklore medicine for the treatment of gastrointestinal disorders such as cholera, diarrhea and dysentery¹⁻⁷. However, several of them have not been investigated from a pharmacological point of view to demonstrate their antibacterial properties, which could support their use as anticholera or antidiarrheal remedies in traditional medicine.

The objective of the present study was to evaluate the antibacterial activities of the extracts of some plants used in Indian traditional healthcare system, against a group of pathogenic bacteria. These plants are used by the indigenous people in different parts of India for the treatment of infectious diseases such as cholera, diarrhea, dysentery and other gastrointestinal disorders.

EXPERIMENTAL

Plant materials

We ethnobotanically selected nine Indian medicinal plants [*Albizia lebbeck* (L.) Benth. (Mimosaceae), *Terminalia chebula* Retz. (Combretaceae), *Syzygium cumini* (L.) Skeels (Myrtaceae), *Solanum nigrum* L. (Solanaceae), *Picrorhiza kurrooa* Royle ex Benth. (Scrophulariaceae), *Butea monosperma* (Lam.) Taub. (Papilionaceae), *Saraca indica* auct.non L. (Leguminosae), *Aegle marmelos* (L.) Correa ex Roxb.

(Rutaceae), and *Withania somnifera* (L.) Dunal (Solanaceae)] for antimicrobial screening in the present study. The plants were collected by the authors from the local area (West Bengal) and another region (Uttaranchal) in India. The botanical identification of the plant samples was carried out by Drs ABD. Selvan and Madhusudan Mandal of Botanical Survey of India (BSI), Central National Herbarium, Botanic Garden, Howrah, India. The voucher specimens are conserved at Central National Herbarium, BSI, Botanic Garden, Howrah, India. Plant species, local names, parts used, and the voucher specimen numbers are listed in Table 1.

Other materials and reagents

Among the clinical strains of *V. cholerae* used in this study, strains NB2 and SG24 belonged to O1 and O139 serotypes, respectively. The other strains used in this study were *V. cholerae* non-O1, non-O139 (strains PC4 and PC65); *Klebsiella pneumoniae* strain PC36; *A. hydrophila* strain PC16; *Escherichia coli* strain PC80 (Enterotoxigenic *Escherichia coli*, ETEC) and *E. coli* strain VT3 (Enterohaemorrhagic *E. coli*, EHEC); *Pseudomonas aeruginosa* ATCC 15442; and *Bacillus subtilis* ATCC 6623. Antimicrobial susceptibility testing was performed by the disc diffusion method⁸ with commercially available disks (HiMedia, Mumbai, India) of ampicillin (A) (10 µg); chloramphenicol (C)(30 µg); co-trimoxazole (Co) (25 µg); ciprofloxacin (Cf)(5 µg); furazolidone (Fz) (100 µg); gentamicin (G)(10 µg); neomycin (N) (30 µg); nalidixic acid (Na) (30 µg); norfloxacin (Nx) (10 µg); streptomycin (S) (10 µg); tetracycline (T) (30 µg).

Preparation of extracts

Plant extracts were prepared by macerating the air-dried plant part (500 g) with 2500 ml of methanol in a Soxhlet apparatus for 18 h⁹. The extract was then filtered through Whatman no. 42 filter paper and concentra-

Table 1: List of plant species with relevant information

Species	Local name (parts of plant used)	Voucher number	Traditional uses ²⁻⁷
<i>A. lebbbeck</i>	Chhatim (Bark)	BSI/CDM/019	Diarrhoea, piles
<i>T. chebula</i>	Haritaki (Fruit)	BSI/CDM/424	Diarrhoea, indigestion, diabetes
<i>S. cumini</i>	Jam (Seed)	BSI/PKB-SA/03	Diarrhoea, dysentery, piles, indigestion, diabetes
<i>S. nigrum</i>	Kakamachi (Fruit)	BSI/PKB-SA/04	Diarrhoea, inflammation
<i>P. kurrooa</i>	Kutki (Rhizomes)	BSI/CDM/315	Cholera, diarrhoea, dyspepsia
<i>B. monosperma</i>	Palash (Flower)	BSI/CDM/063	Diarrhea, piles, inflammation, skin disease
<i>S. indica</i>	Ashok (Flower)	BSI/PKB-SA/09	Gastrointestinal disorder
<i>A. marmelos</i>	Bel (Leaves)	BSI/PKB-SA/10	Cholera, diarrhoea, gastritis, vomiting, diabetes
<i>W. somnifera</i>	Aswagandha (root)	BSI/CDM/458	Diarrhea, dyspepsia, gastrointestinal disorder

ted in a rotary vacuum evaporator (Eyela, Japan). The concentrated extract (30 ml) was freeze-dried at 0.05 mbar pressure and – 60°C for 4 h in a LSL Secroid lyophilizer (Lyolab BII, Aclens-Lausanne, Switzerland). The dried material was stored at - 20°C pending further use. The yield of each extract is shown in Table 1. Test samples were prepared by re-suspending the lyophilized powder in methanol.

Antimicrobial assessment

Isolates were considered susceptible, less susceptible, or resistant to a particular antimicrobial agent on the basis of the diameters of the inhibitory zones that matched the criteria of the manufacturer's interpretation table, which followed the recommendations of the National Committee for Clinical Laboratory Standards¹⁰. The American Type Culture Collection (ATCC) strains, *Escherichia coli* ATCC 25922 and

Staphylococcus aureus ATCC 25923, were used for quality control.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using the broth microdilution method¹¹. An inoculum of the microorganism was prepared from 24 h cultures grown in Mueller-Hinton broth (MHB) (HiMedia) and suspension was adjusted with a turbidity equivalent to that of a 0.5 McFarland standard. The suspension was further diluted 1:10 in sterile MHB to obtain a final inoculum of 5×10^5 CFU/ml. The 96-well round bottom sterile plates were prepared by dispensing 180 µl of the inoculated broth into each well. A 20 µl aliquot of the plant extract was added. The concentrations of plant extract tested were 0.05, 0.125, 0.25, 0.4, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 10, 12, 14, 16, 32 mg/ml. Dilutions of ampicillin served as positive control, while broth with 20 µl of methanol was used as negative control.

Haemolysis test

Haemolysis test was employed to determine cellular toxicity of the extract as previously described¹². Plant extracts at concentrations ranging from 6 to 32 mg/ml, were incubated with an equal volume of 1% human red blood cells in phosphate buffered saline (10mM PBS, pH 7.4) at 37°C for 1 h. Non-hemolytic and 100% hemolytic controls were the buffer alone and the buffer containing 1% Triton X-100, respectively. Cell lysis was monitored by measuring the release of hemoglobin spectrophotometrically at 540 nm.

Phytochemical screening

Phytochemical screening was performed following the method described previously¹³. For total phenolics, 0.2 ml of plant extract (5 mg/ml) was diluted to 3 ml with water and 0.5 ml of Folin-Ciocalteu reagent was added. Following incubation for 3 min at room temperature, 2 ml of 20% w/v Na₂CO₃ was added. The mixture was heated in a boiling water bath for 1 min and allowed to cool to room temperature. It was centrifuged and the content of phenolics in the supernatant was determined spectrophotometrically at 650 nm (model U-2000, Hitachi), and expressed as mg/g of gallic acid equivalents (GAE) per lyophilisate.

For total flavonoids, 0.2 ml of dried extract (5 mg/ml) was diluted to 2 ml with methanol, and 0.1 ml of 10% w/v aluminium chloride, 0.1 ml of 1 mol/lit potassium acetate and 2.8ml of water were added sequentially. The mixture was then incubated at room temperature for 30 min. Following centrifugation, the flavonoids content of the supernatant was measured spectrophotometrically at 415 nm, and expressed as mg/g, based on rutin as standard.

RESULTS

The results of the tests are presented in Tables 2 - 4. The antibiotic susceptibility test of the clinical and environmental strains

included here was performed to confirm their multi-drug resistance patterns. These were found to conform to multi-drug resistant. Antibiotic resistance patterns of *V. cholerae*, strains PC4, PC65, SG24 and NB2 were Fz-A, A-T, Co-S, and T-Na-Fz-Co-S, respectively.

The extracts of *T. chebula* and *S. cumini* had strong bactericidal activity with MIC ranging from 0.125 to 3 mg/ml and MBC ranging from 0.25 to 4 mg/ml, against *V. cholerae* (serotypes O1, O139, and non-O1, non-O139) *A. hydrophila*, and *B. subtilis*; and moderate activity with MIC, 8-12 mg/ml and MBC ranging from 16.0 to 24.0 mg/ml against *P. aeruginosa*, ETEC and EHEC. *S. nigrum* was active against *V. cholerae* and did not show activity against *E. coli* and *P. aeruginosa*. The MBC of *P. kurrooa* extract was between 3 and 32 mg/ml against *V. cholerae* and *A. hydrophila*, but the extract was inactive against *E. coli* and *P. aeruginosa*. The flower extract of *B. monosperma* showed bactericidal activity against *V. cholerae*, *A. hydrophila* and *B. subtilis* with MBC ranging from 1.75 to 8 mg/ml. *W. somnifera* showed strong bactericidal activity (MBC, 2 mg/ml) against *B. subtilis* and moderate activity against *V. cholerae* and *A. hydrophila* with MBC ranging from 16 to 32 mg/ml. *S. indica* evoked strong bactericidal activity against *V. cholerae* and *A. hydrophila* with MBC ranging from 1 to 5 mg/ml while *A. lebeck* and *A. marmelos* had moderate activity against *V. cholerae*, *A. hydrophila* and *B. subtilis*. In addition, the bactericidal activity of *S. indica* against *E. coli* and *P. aeruginosa* was not observed within the concentration ranges of the extracts tested. Except for *S. cumini* (MIC, 24 mg/ml and MBC, 32 mg/ml), all extracts were inactive against *K. pneumoniae*.

Phytochemical screening revealed that the crude methanol extracts contained phenolics (10.6±0.9 - 365.9±16.1 mg/g) and flavonoids (0 - 65.6±2.8 mg/g) (see Table 2). None of the extracts tested in the present study released haemoglobin and hence were not

Table 2: Yield of plant extracts with their phenolic and flavonoid contents

Species	Yield (% w/w)	Total phenolic content ^a	Total flavonoid content ^b
<i>A. lebbeck</i>	5.3	41.9± 0.6	3.0± 1.0
<i>T. chebula</i>	47.6	365.9± 16.1	36.3± 1.8
<i>S. cumini</i>	14.8	231.5±10.3	27.0± 0.9
<i>S. nigrum</i>	8.0	10.6±0.9	7.3± 0.9
<i>P. kurrooa</i>	35.6	56.9± 2.6	6.8± 0.8
<i>B. monosperma</i>	27.0	136.5± 5.6	50.2± 2.3
<i>S. indica</i>	9.3	106.9± 1.2	24.3± 1.9
<i>A. marmelos</i>	11.2	68.0± 2.8	65.6± 2.8
<i>W. somnifera</i>	5.0	11.4±0.5	-

^a Total phenolics content is expressed as gallic acid equivalent (mg of GAE/g of extract).

^b Total flavonoids are expressed as mg of total flavonoid content/g of extract based on Rutin as standard.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts against multi-drug-resistant strains of *V. cholerae* serotypes O1, O139, and non-O1, non-O139

Methanol extract	Antibacterial activity (mg/ml)							
	Strains							
	SG24 (O139)		NB2 (O1)		PC4 (O26)		PC65 (O48)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>A. lebbeck</i>	24.0	24.0	16.0	16.0	16.0	24.0	16.0	16.0
<i>T. chebula</i>	1.5	1.5	1.5	1.5	1.5	2.0	0.125	0.25
<i>S. cumini</i>	1.5	1.8	1.25	1.25	2.5	3.0	2.0	2.0
<i>S. nigrum</i>	-	-	8.0	12.0	-	-	-	-
<i>P. kurrooa</i>	12.0	14.0	8.0	16.0	-	-	2.0	3.0
<i>B. monosperma</i>	4.0	4.0	1.75	1.75	5.0	5.0	2.5	2.5
<i>S. indica</i>	1.5	1.5	3.0	4.0	3.0	4.0	1.0	1.0
<i>A. marmelos</i>	24.0	24.0	24.0	24.0	24.0	24.0	16.0	16.0
<i>W. somnifera</i>	16.0	16.0	16.0	16.0	12.0	16.0	8.0	16.0
Ampicillin	0.016	0.016	0.008	0.008	>1.0	ND	ND	ND

-, MIC/MBC was not obtained within the range of concentration (up to 32 mg/ml) of the extract tested; ND, not determined.

Table 4: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts against *A. hydrophila*, *E. coli* and *B. subtilis*

	Antibacterial activity (mg/ml)							
	<i>A. hydrophila</i> PC16		<i>E. coli</i> VT3 [EHEC]		<i>E. coli</i> PC80 [ETEC]		<i>B. subtilis</i> ATCC 6623	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Methanol extract								
<i>A. lebbeck</i>	24.0	24.0	-	-	-	-	32.0	-
<i>T. chebula</i>	1.0	2.0	12.0	12.0	14.0	16.0	1.0	4.0
<i>S. cumini</i>	1.5	1.5	6.0	-	12.0	16.0	2.5	3.0
<i>S. nigrum</i>	ND	ND	-	-	-	-	ND	ND
<i>P. kurrooa</i>	12	12	-	-	-	-	8.0	-
<i>B. monosperma</i>	8.0	8.0	-	-	-	-	6.0	8.0
<i>S. indica</i>	5.0	5.0	-	-	-	-	1.5	-
<i>A. marmelos</i>	32.0	32.0	-	-	-	-	32.0	32.0
<i>W. somnifera</i>	32.0	32.0	-	-	-	-	2.0	2.0
Ampicillin	0.032	0.032	ND	ND	1.0	1.0	ND	ND

-, MIC/MBC was not obtained within the range of concentration (up to 32 mg/ml) of the extract tested; ND, not determined.

cytotoxic to human erythrocytes at concentrations of up to 32 mg/ml.

DISCUSSION

The extracts of the medicinal plants tested were effective antibacterial agents against a group of microorganisms that are implicated in either cholera, diarrhoea, dysentery or other gastrointestinal disorders. All of these plants had bactericidal activity against at least one of the test microorganisms with MIC ranging between 0.125 and 32 mg/ml and MBC between 0.25 and 32 mg/ml. Among these, *T. chebula* and *S. cumini* showed the most promising broad spectrum antibacterial properties, inhibiting all of the strains tested, especially *V. cholerae*, *A. hydrophila* and *B. subtilis*, with MBC ranging between 0.25 and 4 mg/ml. Additionally, the extracts of *B. monosperma*, *P. kurrooa*, *S. indica* and *S. nigrum* showed strong bactericidal activity

against *V. cholerae*. *W. somnifera*, *B. monosperma*, *A. marmelos* and *S. indica*, and were also active against the Gram-positive bacterium, *B. subtilis*. *T. chebula* showed bactericidal activity against the strain of EHEC, the causative agent of hemorrhagic colitis, dysentery and haemolytic uraemic syndrome (HUS).

It is interesting to note that the extracts of the plants used to treat cholera or diarrhoea in traditional Medicare systems²⁻⁷ were effective against the strains of *V. cholerae*, the causative agents of the dreadful disease, cholera, and cholera-like diseases. To the best of our knowledge, the plant extracts used in this study are being shown for the first time to demonstrate bactericidal activity against enterotoxigenic *Escherichia coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), *A. hydrophila*, and multi-serogroup strains of *V. cholerae*, the causative agents of different

enteropathogenic outbreaks and sporadic diarrhoea. Phytochemical studies revealed that the crude methanol extracts contained phenolics and flavonoids and these compounds have previously been reported to possess antimicrobial activities.

CONCLUSION

The results of this study give some scientific credence to the indigenous uses of the Indian medicinal plants evaluated for the treatment of gastrointestinal disorders such as cholera and diarrheal diseases.

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