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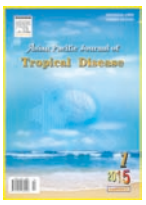

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Antibacterial activity of combined medicinal plants extract against multiple drug resistant strains

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ABSTRACT

Objective: To find out the combined antibacterial efficacy of *Aegle marmelos*, *Aphanamixis polystachya*, *Cuscuta reflexa* and *Aesclynomene indica* against bacterial pathogens.
Methods: Antibacterial potency of combined plant extracts has been tested against *Bacillus subtilis* IFO 3026, *Sarcina lutea* IFO 3232, *Xanthomonas campestris* IAM 1671, *Escherichia coli* IFO 3007, *Klebsiella pneumoniae* ATTC 10031, *Proteus vulgaris* MTCC 321 and *Pseudomonas denitrificans* KACC 32026 by disc diffusion assay. Commercially available standard antibiotic discs were also used to find out antibiotic resistance pattern of test organisms.
Results: Among the test organisms, *Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae* and *Proteus denitrificans* showed resistance against multiple commercially available antibiotics. On the other hand, these multiple drug resistant organisms showed susceptibility against combined plant extracts.
Conclusions: These combined plants extracts showed synergistic antibacterial activity and could lead to new antibacterial drug designing.

1. Introduction

In this era of modern science, with the emergence of new single and multiple drug resistant pathogenic bacterial strains, large number of antibiotic drugs has been developed[1]. But nowadays, new antibiotics are not coming into the market those are competent enough to battle with multiple drug resistant pathogenic bacterial strains[2]. Therefore, scientists are continuously trying to develop new drugs. Plant driven natural compounds can provide potential lead for the development of new drug[3]. Scientists are doing a lot of research on plants regarding this issue[4-6]. From these researches it has been shown that different types of solvent extract of plant show activity against pathogenic bacteria. At present, scientists are investigating to find out antimicrobial synergism within different plant extracts[7] and they found positive results. Rahman *et al*. (2011) showed that combination of aqueous extracts from various spices inhibit the growth of multiple drug resistant *Escherichia coli* (*E. coli*)[7].

The tested bacteria possess the greatest medical significance. The tested organism *Bacillus subtilis* IFO 3026 (*B. subtilis*), *Sarcina lutea* IFO 3232 (*S. lutea*), *Xanthomonas campestris* IAM 1671 (*X. campestris*), *E. coli* IFO 3007, *Klebsiella pneumoniae* ATTC 10031 (*K. pneumoniae*), and *Pseudomonas denitrificans* KACC 32026 (*P. denitrificans*) have been reported as causal organism of some infectious disease in human and plant[7]. *Proteus vulgaris* (*P. vulgaris*) is an opportunistic pathogen and it has been associated with cases of bacteremia, pneumonia and

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focal lesion. It can cause different types of infection including urinary tract infections, wound infections and is a common cause of pyelonephritis and prostatis[8]. *E. coli* and *K. pneumonia* are responsible for various disease including urinary tract, gastrointestinal tract, wound infections, bacteriaemia, pneumonia septicaemia and meningitis[3].

In this study, we use four plants [*Aegle marmelos* (*A. marmelos*), *Aphanamixis polystachya* (*A. polystachya*), *Cuscuta reflexa* (*C. reflexa*) and *Aesclynomene indica* (*A. indica*)] native to Indian subcontinent. All of these plants known to have medicinal properties[9,10]. *A. marmelos* is a very well known and popular medicinal plant and posses antidiarrhoeal, antimicrobial, antiviral, radioprotective, anticancer, chemopreventive, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties[10]. *C. reflexa* is an interesting medicinal plant having antispasmodic, hemodynamic, anticonvulsant, antihypertensive, muscle relaxant, cardiotonic, antiviral, antibacterial, antioxidant, diuretic and hair growth activities[11]. Another plant used in this study *A. indica*, possesses hepatoprotective, anti-inflammatory, antimicrobial and wounds healing effect[12]. Last one used *A. polystachya* is traditionally used in liver and spleen disorders, tumors, ulcer, dyspepsia, intestinal worms, skin diseases, leprosy, diabetes, eye diseases, jaundice, hemorrhoids, burning sensation, arthritis and leucorrhoea[9]. Present study aimed to determine the combined antibacterial effects of these plants against some well known pathogens.

2. Materials and methods

2.1. Plant materials

Leaves of *A. marmelos* (Accession number DACB 38691), *A. polystachya* (Accession number DACB 38688), *C. reflexa* (Accession number DACB 38694) and *A. indica* (Accession number DACB 38689) were collected from Jessore region, Bangladesh during the month of April, 2013 and identified by Bushra Khan, Principal Scientific Officer, National Herbarium, Mirpur, Dhaka 1216, Bangladesh.

2.2. Preparation of extracts

Collected plant materials were water cleaned, manually chopped into small pieces and air dried under shade for fifteen to twenty days. After drying, the plant materials were pulverized into fine powder by a grinding machine and stored in dark airtight container. Then by using these powder and methanol, ethanol, ethylacetate, *n*-hexane, petroleum ether and dichloromethane, crude extracts were prepared. After that, all the extracts were diluted to 4 mg/mL and stored in refrigerator at 4 ℃ in sterile container for further use[5].

2.3. Test organisms

B. subtilis IFO 3026, *S. lutea* IFO 3232, *X. campestris* IAM 1671, *E. coli* IFO 3007, *K. pneumoniae* ATTC 10031, *P. vulgaris* MTCC 321 and *P. denitrificans* KACC 32026 were used in this study. These strains were collected from the Microbiology Laboratory of Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh.

2.4. Antibacterial assay

Commercially available standard antibiotics were used for comparison of the antibacterial activity (Invitrogen, USA). *In vitro* antibacterial activity of the test samples were assayed according to Barry (1980)[13]. Sterile paper disks of 5 mm diameter were impregnated with mixtures of different crude extracts (1:1, 1:1:1, 1:1:1:1) and then air dried. Each disk contains approximately 300 μg of crude extract mixture. All disks were stored at 4 ℃ when not in use[7]. These paper discs were placed on nutrient agar (HiMedia Laboratories, India) inoculated with the test bacteria and incubated at 37 ℃ for 24 h[11]. Nalidixic acid (30 μg/disc) (Invitrogen, USA) were used as positive control and blank discs (impregnated with solvents followed by evaporation) were used as negative control. After incubation the culture plates were examined and the zones of inhibition were measured in millimeter scale[5].

3. Results

All bacterial species showed resistance against ceftazidime (a third generation antibiotic) except *B. subtilis*. Except *S. lutea* and *B. subtilis*, other five strains also showed resistance against cefuroxime. The highest growth inhibition zone was obtained with erythromycin (39 mm, against *B. subtilis*). *E. coli*, *K. pneumonia*e and *P. vulgaris* also showed resistance against ampicillin (Table 1).

Table 2 shows that the combined petroleum ether extract revealed the highest growth inhibition zone against all tested organism except *E. coli*. In case of *E. coli* combined ethanol extract show the highest activity (12.5 mm). *B. subtilis* and *P. denitrificans* showed the highest susceptibility against petroleum ether extract which was 13.5 mm.

Table 1

Antibiotic resistance pattern of test organisms.

Bacterial strain	Inhibition zone (mm)													
	GEN	COT	CXM	CAZ	C	NA	VA	AZM	TE	CIP	COX	E	AMP	CTX
B. subtilis	28.0	20.0	20.0	22.0	36.0	23.0	28.0	27.0	27.0	30.0	28.0	39.0	6.0	24.0
S. lutea	34.0	13.0	110	-	19.0	25.0	15.0	35.0	35.0	30.0	12.0	30.0	16.0	25.0
X. campestris	19.0	7.0	-	-	19.0	20.0	-	28.0	16.0	33.0	-	15.0	11.0	24.0
E. coli	29.0	22.0	-	-	25.0	21.0	21.0	28.0	31.0	29.0	20.0	23.0	-	34.0
K. pneumoniae	20.0	25.0	-	-	25.0	17.0	20.0	27.0	20.0	28.0	-	20.0	-	32.0
P. vulgaris	21.0	20.0	-	-	25.0	20.0	16.0	23.0	21.0	29.0	17.0	18.0	-	20.0
P. denitrificans	29.0	15.0	-	-	24.0	20.0	16.0	20.0	25.0	27.0	16.0	16.0	6.7	17.0

GEN: Gentamicin (10 μg/disc); COT: Co-trimoxazole (25 μg/disc); CXM: Cefuroxime (30 μg/disc); CAZ: Ceftazidime (30 μg/disc); C: Chloramphenicol (30 μg/disc); NA: Nalidixic acid (30 μg/disc); VA: Vancomycin (30 μg/disc); AZM: Azithromycin (30 μg/disc); TE: Tetracycline (30 μg/disc); CIP: Ciprofloxacin (5 μg/disc); COX: Cloxacilin (1 μg/disc); E: Erythromycin (15 μg/disc); AMP: Ampicillin (25 μg/disc); CTX: Cefotaxime (30 μg/disc).

Table 2

Combinatorial effect of methanol, ethanol, ethyl acetate, *n*-hexane, dichloromethane, petroleum ether extract of plants.

Bacterial strain	Inhibition zone (mm)					
	M1/M2/M3	E1/E2/E3/E4	Ea1/Ea2/Ea3/Ea4	H1/H2/H3/H4	P1/P2	D1/D2/D4
B. subtilis	10.0	10.5	8.5	8.0	13.5	8.0
S. lutea	8.5	11.0	8.0	7.0	12.0	7.0
X. campestris	7.0	7.5	6.5	6.5	12.5	8.0
E. coli	9.0	12.5	6.5	7.5	12.0	8.0
K. pneumonia	7.0	10.0	7.0	9.0	10.0	6.5
P. vulgaris	8.0	8.5	7.0	6.5	13.0	7.0
P. denitrificans	8.0	10.0	10.0	7.5	13.5	7.0

M: Methanol; E: Ethanol; Ea: Ethyl acetate; H: *n*-Hexane; P: Petroleum ether; D: Dichloromethane; 1: *A. marmelos*; 2: *A. polystachya*; 3: *C. reflexa*; 4: *A. indica*.

Table 3 shows the antibacterial activity result of different solvent extract combinations. When extracts were combined into 1:1:1:1 ratio, ethyl acetate extract containing combinations showed the highest antibacterial efficacy. The highest growth inhibition zone was detected against *E. coli* by D1/M3/H2/Ea4 combination. The lowest inhibition zone was observed with Ea1/Ea3/D2/D4 extract against *S. lutea*.

These plants individually show low antibacterial activity such as antibiotic activity of *A. indica* had been shown 3.3±2 mm against *Klebsiella* sp. However, in this study, combined extract of *A. indica* with other three plants showed 6.5 to 14.5 mm inhibition zone against *K. pneumonia* which depends on the type extract and solvent of extraction.

4. Discussion

Combined plant extracts therapy would be a potential way for producing desirable synergistic effects to combat bacterial infection as well as to prevent the continuous emergence of bacterial resistance[14]. Microorganisms exhibit a range sensitivity against different extracts, which depends on the type of extract and the solvent of extraction[15]. In this study, *E. coli*, *K. pneumoniae*, *X. campestris*, *P. vulgaris* and *P. denitrificans* demonstrated multiple drug resistance which are in good accord with those found in other studies[7,16]. On the other hand, D1/M3/H2/Ea4 combination extract demonstrated the highest potential effect against *E. coli*. Previous study also showed significant antibacterial activity against *E. coli* and *K. pneumoniae* in P/D/E, P/E and P/D extract combinations[3].

Present study exerted a broad spectrum antimicrobial activity by significantly inhibiting both the Gram-positive and Gram-negative bacteria. Tested organisms showed resistance against third generation antibiotic whereas tested plant extract showed noteworthy activity. *X. campestris* is a plant pathogen for cabbage and cauliflower and *E. coli* and *K. pneumonia* are human pathogen[5]. *E. coli* and *K. pneumonia* are responsible

Table 3

Synergistic activity of various plant extract in different combinations.

Bacterial strain	Inhibition zone (mm)																				
	D1/M3/H2/Ea4	Ea1/M2/E3/D4	E1/P2/Ea3/H4	M1/Ea2/H3/E4	H1/E2/M3/Ea4	P1/D2/E3/Ea4	P1/P2/H3/D4	E1/H2/M3/Ea4	D1/D2/Ea3/E4	M1/M2/E3/H4	Ea1/E2/H3/D4	P1/Ea2/H3/H4	D1/H2/Ea3/Ea4	M1/M3/Ea2/Ea4	E1/E3/D2/D4	Ea1/Ea3/M2/E4	P1/P2/H3/H4	H1/H2/E3/E4	M1/M3/D2/D4	P1/P2/Ea3/Ea4	Ea1/Ea3/D2/D4
B. subtilis	13.0	10.0	12.0	13.0	11.0	11.0	11.0	10.0	10.0	9.0	10.5	8.0	9.0	7.0	7.0	8.0	7.0	10.0	8.0	14.0	7.5
S. lutea	12.0	10.5	9.0	15.0	12.0	11.0	11.5	10.0	13.5	9.0	12.0	7.5	8.0	10.0	10.0	11.0	9.0	10.0	11.0	13.0	6.2
X. campestris	10.0	9.0	12.0	12.0	9.0	10.0	10.0	10.0	11.0	10.0	11.0	7.0	8.5	7.0	8.0	6.5	10.5	9.0	6.5	10.0	6.5
E. coli	15.5	14.0	9.5	15.0	11.0	13.0	10.5	9.0	10.0	8.5	10.0	6.5	7.5	8.0	8.0	8.0	9.0	10.0	9.0	9.0	8.0
K. pneumonia	11.0	8.0	9.0	10.0	14.5	8.0	7.0	7.5	11.0	10.5	10.0	8.0	8.5	11.0	8.0	10.0	10.0	9.5	8.5	9.0	7.0
P. vulgaris	10.0	9.0	8.0	11.0	10.0	9.5	12.0	10.0	12.0	8.5	12.0	7.0	7.5	8.0	7.0	8.0	9.0	10.0	11.0	14.0	7.5
P. denitrificans	12.0	9.0	9.0	10.5	9.0	10.0	9.5	9.0	11.0	10.0	10.0	6.5	8.0	6.5	7.0	8.5	14.5	9.0	11.0	14.0	9.0

M: Methanol; E: Ethanol; Ea: Ethyl acetate; H: *n*-Hexane; P: Petroleum ether; D: Dichloromethane; 1: *A. marmelos*; 2: *A. polystachya*; 3: *C. reflexa*; 4: *A. indica*.

for various disease including urinary tract, gastrointestinal tract, wound infections, bacteriaemia, pneumonia septicaemia and meningitis[3]. This study signifies the effectiveness of combinatorial effect of these plants against both plant pathogen and human pathogen by remarkably inhibiting the growth of these bacteria which provides breakthrough potential source for treatment of such disease. The largest inhibition zone was found on human pathogen *E. coli*.

It is believed that plant show antibacterial activity due to the presence of large number of constitutive plant components including phenols, unsaturated lactones, saponin, cyanogenic glycosides, glucosinolates and tannins[7]. We didn't perform any phytochemical analysis in this study. So this study is regarded as preliminary step and it suggests that further detailed study is needed to evaluate valuable efficacies of these plant extract.

From the consequence of the present study it can be postulated that these plant extract possess some compounds which may be linked to the antibacterial activity. So it is urged that this synergistic effect leads to new choice for antibacterial drug designing. However, additional study could be carried out to isolate and characterize their active compounds as well as to find out the actual mechanism of antibacterial activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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