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Synergistic antibacterial effects of three edible plants extract against antibiotic-associated diarrheagenic resistant bacteria

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Abstract

In vitro synergistic antibacterial effects among *Alocasia macrorrhizos* rhizome, *Amorphophallus paeoniifolius corm* and *Colocasia esculenta corm* extracts were tested against six resistant bacteria viz., *Yersinia enterocolitica, Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Clostridium difficile* and *Staphylococcus aureus*. The inhibition zone was compared with the commercially available antibiotic (tetracycline). High inhibitory activity was observed against *E. coli* (12.67±0.33 mm) and *S. aureus* (12.50±0.29 mm) for methanol extract at 800 $mgml^{-1}$ of concentration. MIC and MBC of the extracts ranged from 200-580 $mgml^{-1}$ and 250-650 $mgml^{-1}$ respectively. The lowest MIC and MBC of the extracts were measured against *E. coli.*

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Introduction

Diarrheal diseases caused by bacterial pathogens are a major problem worldwide, especially in developing countries (Wilson *et al*., 2006; Jousilahti *et al*., 1997). Antibiotic-associated diarrhea (AAD) may be the most common adverse clinical effect of antibiotic-mediated disruption of the gastrointestinal microflora (Beaugerie and Petit, 2004). The spectrum of AAD ranges from the nuisance of frequent loose stools to fulminate, life threatening colitis. The incidence of AAD differs with the antibiotic and varies between 5% and 25% (Bartlett and John, 1996). The medical literature reports that 5 to 39% of adults (McFarland, 1998; Wistrom *et al*., 2001) and 11 to 40% of children (Elstner *et al*., 1983; Turck *et al*., 2003; Kotowska *et al*., 2005) will suffer from diarrhea when treated with antibiotics.

The discovery of antibiotics in the early twentieth century provided an increasingly important tool to combat bacterial diseases. As antibiotics are increasingly used and misused, the bacterial strains become resistant to antibiotics rapidly (Abeysinghe and Wanigatunge, 2006). Due to the increase of resistance to antibiotics, there is a pressing need to develop new and innovative antimicrobial agents. Among the potential sources of new agents, plants have long been investigated. Because, they contain many bioactive compounds that can be of interest in therapeutic (Djeussi *et al*., 2013). The use of plant extracts as well as other alternative forms of medicinal treatments, is enjoying great popularity in late 1990s (Cowan, 1999). Plants used for traditional medicines contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases (Ofokansi *et al*., 2011; Diallo *et al*.,1999). Many phytomedicines exert their beneficial effects through the additive or synergistic action of several chemical compounds acting at single or multiple target sites (Briskin, 2000).

Both food and medicinal plants have interventional uses. This exists mainly in indigenous and local traditions. Food can be used as medicine and vice versa. However, certain wild edible plants are used because of their assumed health benefits and thus can be called medicinal foods (Etkin, 1994). Wild edible plants have always been important in the folk traditions. However, food and medicinal uses of these plants have been two of the most relevant and consistent reasons for popular plant management (Abbasi *et al*., 2013). Therefore, in the study we used three edible plants and focused at investigating the synergistic antibacterial potentials of such plants against antibiotic-associated diarrheagenic resistant bacteria.

Materials and Methods

Collection of plant materials and antibiotics

*Alocasia macrorrhizos (*Family-Araceae*)* rhizome, *Amorphophallus paeoniifolius (*Family-Araceae*) corm* and *Colocasia esculenta* (Family-araceae) corm were collected from Binodpur Bazar, Rajshahi, Bangladesh in March 2012. Any type of adulteration was strictly avoided during collection. These plants were identified and authenticated by Mr. A.H.M. Mahbubur Rahman, Associate professor and plant taxonomist, Department of Botany, University of Rajshahi, Bangladesh. Commercial antibiotic tetracycline (Square Pharmaceuticals Ltd., Bangladesh) was used during antibacterial study.

Preparation of plant extracts

Collected *A. macrorrhizos* rhizomes, *A. paeoniifolius corms* and *C. esculenta corms* were washed with clean sterile distilled water, cut into small pieces and dried for 3 days in oven under 60°C to reduce water content. Then the dried plant material pieces were crushed into fine powder using mortar, pestle and electric blender (Nokia, Osaka-Japan). Five-gram dried powder (1:1:1) from each plant was dipped into 100ml of different organic solvents (methanol and ethanol) separately into a conical flask followed by air

tight with rubber corks, and left for 2 days on orbital shaking (IKA Labortechnik KS 250 Basic Orbital Shaker, Staufen, Germany). The well refined solution was filtrated through Teton cloth and Whatman No. 1 filter paper in a beaker followed by evaporation of solvent using water bath (4 holes analogue, Thermostatic water bath, China) until formation of semisolid extract. Semi solid extracts were dissolved into respective solvent and preserved in airtight screw cap tube at 4°C for further use.

Bacterial species used

The synergistic antibacterial activity among *A. macrorrhizos* rhizomes, *A. paeoniifoliu corms*, *C. esculenta* corms were assayed against six resistant bacteria viz., *Yersinia enterocolitica*, *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Clostridium difficile*. and *Staphylococcus aureus* isolated from antibiotic-associated diarrhea affected patients.

Antibacterial assay

In vitro synergistic antibacterial activity among *A. macrorrhizos* rhizomes, *A. paeoniifolius corms* and *C. esculenta corms* extracts as well as antibiotics were tested against six studied bacteria using agar disc diffusion method (Parekh and Chanda, 2007). Under aseptic conditions, sterilized Whatman no. 1 filter paper discs (6 mm in diameter) were impregnated with 10 µl of different concentration (200, 400, 600 and 800 $mgml^{-1}$) of extracts followed by air-drying and placed on seeded nutrient agar plates. 30 µl of bacterial suspension ($10^8 cfuml^{-1}$) was used for preparing seeded nutrient agar plates. Negative controls were prepared using respective solvents. The Petri-plates were incubated at 37°C for 24h. After incubation, antibacterial activity was determined by measuring the zone of inhibition in millimeter scale (including disk) against the studied bacteria. The results were compared with standard or broad-spectrum antibiotics (Tetracycline at 0.03µgml^{-1}) as a positive control. Each assay was carried out in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC of plant extracts were determined according to Doughari *et al.* (2007). Different concentration (150, 180, 200, 220, 250, 280, 300, 320, 350, 380, 400, 420, 450, 480, 500, 520, 550, 580, 600, 620 and 650 $mgml^{-1}$) of extracts were used for MIC determination. 0.5 ml of varying concentration of these extracts was taken into test tubes and 2 ml nutrient broth was added separately, finally a loop-full of the test bacteria ($10^8 cfuml^{-1}$) were introduced in those test tubes. Only bacterial suspension containing test tubes with nutrient broth instead of the extracts were used as a control. The culture tubes were incubated at 37°C for 24 hours. After incubation, the lowest concentration of extracts that inhibited visible growth of studied bacteria in test tubes was taken as MIC. To determine the MBC, a loop-full bacterium was collected from MIC position and sub cultured onto nutrient agar plates. Petri dishes were incubated at 37°C for 24 hours. The lowest concentrations of extracts with no visible growth of studied bacteria on agar plates were recorded as MBC.

Statistical analysis

Statistical analysis (ANOVA) was done using CropStat 7.2. Least Significant Difference (LSD) test was used to speculate further if there was a significant difference within extracts, various concentrations, studied bacteria and interaction effect between them. P values <0.05 were considered as significant.

Results

Antibacterial assay

The results of synergistic antibacterial effect of combination extracts are shown in Table 1. The studied concentrations of extract exhibited different degrees of antibacterial activity depend on bacterial strains and solvents. The zone of inhibition was ranged from 0.00 to 12.67±0.33 mm in methanol extracts and 0.00 to 11.67±0.14

mm in ethanol extracts. For methanol extract, the higher inhibition zones 12.67±0.33 and 12.50±0.29 mm were measured at 800 mg/ml against *E. coli* and *S. aureus* respectively. On the other hand, the higher inhibition zones 11.67±0.14 and 11.33±0.33 mm were measured at 800 mg/ml against *E. coli* and *S. aureus* respectively in ethanol extracts. Methanol extract, displayed a relatively better antibacterial effect against tested bacteria. In all cases, the activity of the extracts was compared with antibiotic (Tetracycline). Statistical results showed significant differences ($P<0.05$) among the bacterial strains, concentrations and extracts, and their interaction cases R×B, C×B, R×C×B, E×B, E×C, E×C×B (Table 2). According to the LSD test results (Table 3), means of strain, significant differences were observed among *E. coli, P. aeruginosa, C. difficile* and *S. aureus*. But no significant difference was observed between *Y. Enterocolitica* and *K. pneumoniae*. Highest and lowest mean value was found against *E. coli* and P*. aeruginosa* respectively. In case of means of concentration, significant difference was observed among them, highest for 800 $mgml^{-1}$ (5.41667) followed by 600 $mgml^{-1}$ (3.23611), 400 $mgml^{-1}$ (1.47222), 200 $mgml^{-1}$ (0.12500) as expected.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC results of extracts are presented in Table 2. The MIC value of methanol extracts was ranged from 200 (*E. coli)* to 420 (*P. aeruginosa*) $mgml^{-1}$. In case of the MIC value of ethanol extracts was ranged from 400 (*E. coli*, *C. difficile*, *S. aureus*) to 580 (*P. aeruginosa*) $mgml^{-1}$. In two types of extract, methanol extract gave lowest MIC value (200 $mgml^{-1}$) followed by ethanol (400 $mgml^{-1}$). Moreover, in methanol extracts, MBC values were ranged from 250 (*E. coli)* to 480 (*P. aeruginosa*) $mgml^{-1}$. On the other hand, in ethanol extracts, MBC values were ranged from 450 (*E. coli*) to 650 (*P. aeruginosa*) $mgml^{-1}$. In two types of extract, methanol extracts gave lowest MBC value (250 $mgml^{-1}$).

Table 1. Synergistic antibacterial effects among *A. macrorrhizos* rhizomes, *A. paeoniifolius corms* and *C. esculenta corm* extracts.

Extract ($mgml^{-1}$)	Solvent	Bacteria						
		Y. enterocolitica	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***C. difficile***	***S. aureus***	
200	ME	0.00	7.50±0.29	0.00	0.00	0.00	0.00	Zone of inhibition (mm)
	ET	0.00	0.00	0.00	0.00	0.00	0.00	
400	ME	7.67±0.33	8.17±0.14	7.67±0.14	7.33±0.33	8.00±0.00	8.33±0.33	
	ET	7.17±0.14	7.33±0.17	7.17±0.44	0.00	7.50±0.29	7.33±0.14	
600	ME	9.33±0.14	10.33±0.33	9.50±0.29	9.17±0.44	9.67±0.33	9.83±0.27	
	ET	8.83±0.33	9.50±0.29	8.33±0.33	8.33±0.33	9.00±0.00	9.50±0.29	
800	ME	11.33±0.14	12.67±0.33	11.17±0.44	11.50±0.29	11.67±0.14	12.50±0.29	
	ET	10.67±0.33	11.67±0.14	10.83±0.14	10.50±0.29	10.83±0.14	11.33±0.33	
0.03	TET	12.17±0.14	13.83±0.17	12.00±0.47	11.67±0.33	11.83±0.27	12.67±0.33	

ET= Ethanol extract, ME = Methanol extract, TET = Tetracycline. Data were represented mean of zone inhibition (mm) ± SEM of three replicates.

Table 2. Statistical results (ANOVA) of the synergistic antibacterial effects among *A. macrorrhizos* rhizome, *A. paeoniifolius corm* and *C. esculenta corm* extracts.

Source of variation	Degree of Freedom	Sum of squares	Mean Squares	F Value	Probability
Replication (R)	2	0.510416	0.255208	4.08	0.023
Bacteria (B)	5	18.7917	3.75833	60.13	0.000
R×B	10	2.13542	0.213542	3.42	0.002
Concentrations (C)	5	566.285	188.762	****	0.000
C×B	15	6.52778	0.435185	6.96	0.000
R×C×B	36	4.68750	0.130208	2.08	0.009
Extracts (E)	1	14.0625	14.0625	225.00	0.000
E×B	5	1.45833	0.291667	4.67	0.002
E×C	3	2.11806	0.706019	11.30	0.000
E×C×B	15	3.86111	0.257407	4.12	0.000
R× E×C×B	48	3.00000	0.625000	1.00	0.500
Total (Corrected)	143	623.438	4.35970		

*Indicates very high value

Table 3. Analysis of mean data of the synergistic antibacterial effect among *A. macrorrhizos* rhizome, *A. paeoniifolius corm* and *C. esculenta corm* extracts.

Variables	Growth inhibition diameter (mm)
Bacteria	
Y. enterocolitica	2.37500 d
E. coli	3.18750 a
K. pneumoniae	2.29167 d
P. aeruginosa	2.10417 e
C. difficile	2.58333 c
S. aureus	2.83333 b
LSD	0.145094
Concentrations	
200 $mgml^{-1}$	0.12500 d
400 $mgml^{-1}$	1.47222 c
600 $mgml^{-1}$	3.23611 b
800 $mgml^{-1}$	5.41667 a
LSD	0.118469
Extracts	
Methanol	2.87500 a
Ethanol	2.25000 a
LSD	0.837702

Means followed by different letter (S) down the column are significantly different among the strains, concentration as well as extracts at $p<0.05$. Here any two means having a common letter are not significantly different at the 5% level of significance.

Table 4. MIC and MBC of *A. macrorrhizos* rhizome, *A. paeoniifolius corm* and *C. esculenta corm combination* extracts.

Sl. No.	Bacteria	MIC value ($mgml^{-1}$)		MBC value ($mgml^{-1}$)	
		ME	ET	ME	ET
1	*Y. enterocolitica*	400	420	450	480
2	*E. coli*	200	400	250	450
3	*K. pneumoniae*	400	420	450	480
4	*P. aeruginosa*	420	580	480	650
5	*C. difficile*	380	400	450	480
6	*S. aureus*	380	400	450	480

Discussion

The results showed that the methanol combination extracts of *A. macrorrhizos* rhizome, *A. paeoniifolius corm* and *C. esculenta corm* had the best antibacterial activity. The highest activity recorded against *E. coli* and the nearest activity recorded against *S. aureus*. The lowest MIC and MBC values observed for *E. coli* is a good indication of high efficacy against this bacteria and high MIC and MBC values are indication of low activity (Doughari *et al.,* 2007). The highest MIC and MBC values suggest that the extracts are less susceptible. In this study, extracts showed different degrees of growth inhibition depending upon the bacterial strains. These variations were found because strains are genetically different from each other, and this is probably due to the differences in chemical composition and structure of the cell wall of both types of microorganisms (Kaushik and Goyel, 2008). Increasing of the concentrations level of extracts had a significant ($P<0.05$) inhibitory effect on all studied bacteria. Extracts prepared in methanol extract gave better activity than ethanol extracts, and it could be better solubility of active components in methanol. It has been reported that different phytoconstituents have different degrees of solubility in different types of solvents depending on their polarity (El-Mahmood and Doughari, 2008). Moreover, several authors have conducted antibacterial study using methanol as an extraction solvent and shown comparatively better activity than others (El-mahmood and Ameh, 2007; Nataraj *et al*., 2009; Prasannabalaji *et al*., 2012). No studies on the effects of various combinations of plants extracts were previously conducted to determine an antibacterial activity suitable for incorporating in foods (Sagdic *et al*., 2005). Study revealed that Several investigators conducted related investigation and recommend *A. paeoniifolius* extracts as a source of antibacterial agent (Nataraj *et al*., 2009; Dubey *et al.*, 2010). Research has shown that extract of *C. esculenta* corms exhibit antimicrobial activity (Dubey *et al.*, 2010).

Conclusion

The extract showed good antibacterial activity against all the tested bacteria. The synergistic effect from the association of different plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective plants when it is no longer effective by itself during therapeutic treatment.

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References

Abbasi AM, Khan MA, Zafar M. 2013. Ethno-medicinal assessment of some selected wild edible fruits and vegetables of lesser-himalayas, Pakistan Journal of Botany **45(SI)**, 215-222.

Abeysinghe PD, Wanigatunge RP. 2006. Evaluation of antibacterial activity of different mangrove plant extracts. Ruhuna Journal of Science **1**, 104-112.

Bartlett, John G. 1996. Management of *Clostridium difficile* infection and other antibiotic-associated diarrhoeas. European Journal of Gastroenterology & Hepatology **8**, 1054-61.

Beaugerie L, Petit JC. 2004. Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. Best Practice & Research Clinical Gastroenterology **18**, 337-52.

Briskin DP. 2000. Medicinal plants and phytomedicines. Linking plant Biochemistry and Physiology to Human Health. Plant Physiology **124(2),** 507-514.

Cowan MM. 1999. Plant products as antimicrobial agents. Clinical microbiology reviews **12(4),** 564-582.

Diallo D, Hveem B, Mahmoud MA, Berge G, Paulsen BS, Maiga A. 1999. An ethnobotanical survey of herbal drugs of Gourma district, Mali. Pharmaceutical Biology **37(1)**, 80-91.

Djeussi DE, Noumedem JAK, Seukep JA, Fankam AG, Voukeng IK, Tankeo SB, Nkuete AHL, Kuete V. 2013**.** Antibacterial activities of selected edible plants extracts against multidrug-resistant gram-negative bacteria. Biomed central complementary and alternative medicine **13**, 164.

Doughari JH, Elmahmood AM, Manzara S. 2007. Studies on the antibacterial activity of root extracts of *Carica papaya* L. African Journal of Microbiology Research **1(3),** 37-41.

Dubey A, Mishra N, Singh N. 2010. Antimicrobial activity of some selected vegetables. International Journal of Applied Biology and Pharmaceutical Technology **1(3)**, 994.

El-mahmood AM, Ameh JM. 2007. *In vitro* antibacterial activity of *Parkia biglobosa* (Jacq) root bark extract against some microorganisms associated with urinary infections. African Journal of Biotechnology **6(11),** 1272-1275.

El-Mahmood AM, Doughari JH. 2008. Phytochemical screening and antimicrobial evaluation of the leaf extracts of *Cassia alata* Linn. African Journal of Pharmacy and Pharmacology **2(7)**, 124-129.

Elstner CL, Lindsay AN, Book LS, Matsen JM. 1983. Lack of relationship of Clostridium difficile to antibiotic-associated diarrhoea in children. The Pediatric Infectious Disease Journal **2(5)**, 364-66.

Etkin N. 1994. The cull of the wild. In Eating on the Wild Side: The Pharmacologic, Ecologic, and Social Implications of Using Non cultigens. Edited by: Etkin NL.Tucson/London: University of Arizona Press p.1-21.

Jousilahti P, Madkour SM, Lambrechts T Sherwin E. 1997. Diarrhoeal disease morbidity and home treatment practices in Egypt. Public Health **111,** 5-10.

Kaushik P, Goyel P. 2008. *In vitro* evaluation of *Datura innoxia* (thorn-apple) for potential antibacterial activity. Indian Journal of Microbiology **48(3),** 353-357.

Kotowska M, Albrecht P Szajewska H. 2005. *Saccharomyces boulardii* in the prevention of antibiotic-associated diarrhoea in children: a randomized double-blind placebo-controlled trial. Alimentary Pharmacology & Therapeutics **21(5)**, 583-590.

McFarland LV. 1998. Epidemiology, risk factors and treatments for antibiotic-associated diarrhea. Digestive Diseases **16,** 292-307.

Nataraj HN, Murthy RLN, Setty SR. 2009 *In vitro* screening of antimicrobial activity and preliminary phytochemical screening of Suran. Pharmacologyonline **1**, 189-194.

Ofokansi KC, Eze AO, Uzor PF. 2011. Evaluation of the antimicrobial activity of the aqueous and methanolic leaf extracts of *Mitacarpus villosus* with amoxicillin. African Journal of Pharmacological Research **3(1),** 43-47.

Parekh J, Chanda SV. 2007. Antibacterial activity of aqueous and alcoholic extracts of 34 Indian medicinal plants against some *Staphylococcus* Species. Turkish Journal of Biology **32(1),** 63-71.

Prasannabalaji N, Muralitharan G, Sivanandan RN, Kumaran S, Pugazhvendan SR. 2012. Antibacterial activities of some Indian traditional plant extracts. Asian Pacific Journal of Tropical Disease p.291-295.

Sagdic O, Yasar S, Kisioglu AN. 2005. Antibacterial effects of single or combined plant extracts. Annals of microbiology **55(1)**, 67-71.

Sivam GP, Lampe JW, Ulness B, Swanzy SR, Potter JD, 1997. *Helicohacter pylori in vitro* susceptibility to garlic (*Allium sativum*) extract. Nutrition and Cancer **27(2),** 118-121.

Turck D, Bernet JP, Marx J, Kempf H, Walbaum O, Lacombe A, Rembert F, Toursel F, Bloch K. 2003. Incidence and risk factors of oral antibiotic-associated diarrhea in an outpatient pediatric population. Journal of Pediatric Gastroenterology & Nutrition **37(1)**, 22-26.

Wilson G, Easow JM, Chiranjoy M Shivananda PG. 2006. Isolation and antimicrobial susceptibility of *Shigella* from patients with acute gastroenteritis in western Nepal. Indian Journal of Medical Research **123(2)**,145-50.

Wistrom J, Norrby SR, Myhre EB, Eriksson S, Granstrom G, Lagergren L, Englund G, Nord CF, Suvenungsson B. 2001. Frequency of antibiotic-associated diarrhea in 2462 antibiotic-treated hospitalized patients: a prospective study. Journal of Antimicrobial Chemotherapy **47(1)**, 43-50.