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In vitro controlling of selected human diarrhea causing bacteria by clove extracts (*Syzygium aromaticum* L.)

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Abstract

Antibacterial activity of clove extracts (*Syzygium aromaticum* L.) was proven against five diarrhea causing bacteria. This was further confirmed when compared with commonly used three commercial antibiotics (ciprofloxacin, tetracycline and erythromycin) as a positive control. Significant differences ($P<0.0001$) were observed in the effect of the antimicrobial agents (clove extracts and antibiotics), and in the sensitivities of the bacterial species ($P<0.0001$) to the antimicrobial agents. Clove extracts had significant ($P<0.001$) activity with the acetone extract demonstrating highest activity followed by antibiotics and other extracts against tested bacteria. The zone of inhibition of clove extracts was ranged from 7.33 to 12.00 mm whereas in antibiotics, it was 0.00 to 11.67 mm. Of all the bacteria, *Salmonella typhimurium* was the most susceptible against all of the extracts as well as concentrations of clove, while low MIC (180 $mgml^{-1}$) and MBC (680 $mgml^{-1}$) of the extracts were observed against *Shigella dysenteriae*. Consequently, clove has a significant antidiarrheal activity and it could be used as an effective antibacterial agent, alternative to the use of antibiotics.

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Introduction

In developing countries diarrhea is the most common causes of morbidity and mortality (Amstrong and Cohen, 1999) and it caused several million of deaths in the world annually (Field, 2003). Many bacteria, virus and protozoa have been isolated from diarrhea patients, especially *Salmonella typhimurium*, *Escherichia coli*, *Shigella dysenteriae*, *Proteus mirabilis* (Prescott *et al.,* 2005; Eja *et al.,* 2007), *Yersinia enterocolitica* (Okwori *et al.,* 2007), *Vibrio cholera* (Zuckerman *et al.,* 2007), *Campylobacter jejuni*, *Clostridium difficile* (Prescott *et al.,* 2005) etc. bacteria are responsible for acute and chronic diarrhea. Antibiotics are the essential part for combating harmful bacterial infections *in vivo* (Kaushik and Goyel, 2008), but repeated and improper uses of antibiotics resulting drug-resistant bacteria. To overcome this problem an alternative therapy is very much needed and researchers are looking for developing alternative strategies (Sivam *et al.,* 1997). The World Health Organization (WHO) has included a programme for the control of diarrhea, which involves the use of traditional herbal medicine (Snyder and Merson, 1982). Various herbs and spices have been recognized by their medicinal value used as an alternative antimicrobial agent to antibiotics, and several plants have been reported to be used in treating and managing diarrhea diseases (Agunu *et al.,* 2005). Cloves (*Syzygium aromaticum* L.) are the aromatic dried flower buds, and several studies have demonstrated on potent antibacterial effects of clove (Cai and Wu, 1996; Bae *et al.,* 1998; Li *et al.,* 2005; Fu *et al.,* 2007). The present study was undertaken for *in vitro* controlling of human diarrhea causing bacteria by clove extracts using agar disc diffusion assay.

Materials and methods

Collection of plants and Antibiotics

Locally available flower buds of *Syzygium aromaticum* L. (Common name: Clove, Family: Myrtaceae) and three commercial antibiotics namely ciprofloxacin, tetracycline and erythromycin (Beximco Pharmaceuticals Ltd., Bangladesh) were used during antibacterial study.

Collection of bacterial isolates

Five bacteria with the accession number *Salmonella typhimurium* BMLRU1021, *Escherichia coli* BMLRU1023, *Shigella dysenteriae* BMLRU1025, *Proteus mirabilis* BMLRU1027 and *Yersinia enterocolitica* BMLRU1029 were used in this study. These bacterial strains were isolated and identified from stool and urine samples (diarrhea associated) according to Holt *et al.* (1994) using their respective standard strain (collected from ICDDRB, Dhaka, Bangladesh) in Biotechnology and Microbiology laboratory, Department of Botany, University of Rajshahi, Bangladesh.

Preparation of crude extracts

Collected clove flower buds were dried for 3 days in oven under 60°C then crushed into fine powder using mortar, pestle and electric blender (Nokia, Osaka-Japan). Ten-gram dried powder of clove was dipped into 100ml of different organic solvents (methanol, ethanol and acetone) 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.

Preparation of antibiotics

Antibiotics solution was prepared as described by Ekwenye and Elegalam (2005). The commercial antibiotics ciprofloxacin (500 mg), tetracycline (500 mg) and erythromycin (250 mg) were crushed manually using mortar and pestle. Ciprofloxacin and tetracycline were dissolved in 10 ml de-ionized distill

water separately, and erythromycin was dissolved in 10 ml ethanol (95%). The solutions were preserved at 4°C until further use.

Antibacterial assay

In vitro antibacterial activity of clove extracts as well as antibiotics were tested against five 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 solvent extracts (200, 400, and 600 $mgml^{-1}$) as well as antibiotics (0.02, 0.04 and 0.06 $mgml^{-1}$) followed by air-drying and placed on seeded nutrient agar plates. 30 µl of bacterial suspension (10^8 cfu ml^{-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 against the studied bacteria. 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). For MIC determination, 0.5 ml of varying concentrations of the extracts (150, 180, 200, 220, 250, 280, 300, 320, 350, 380, 400, 420, 450, 480, 500, 520, 550, 580, 600, 620 and 650 $mgml^{-1}$) were added with nutrient broth (2 ml) in test tubes, then a loop-full of the test bacteria (10^8 cfu ml^{-1}) was introduced. A tube containing nutrient broth was seeded only with the test bacteria, as described above, to serve as control. The culture tubes were incubated at 37°C for 24 h. After incubation, the tubes were examined for microbial growth by observing for turbidity.

To determine the MBC, for each set of test tubes in the MIC determination, a loopful of broth was collected from those tubes that did not show any growth and inoculated onto sterile nutrient agar by streaking. All the plates were then incubated at 37°C for 24 h. After incubation the concentration at which no visible growth was seen, noted as MBC.

Statistical analysis

Statistical analysis (ANOVA) was performed using software SPSS (version 10.0; SPSS Inc., Chicago IL, USA) and MSTAT (version 2.10; Russell, D. Freed, Michigan State University, USA) and expressed as mean ± SEM. Least Significant Difference (LSD) test was used to speculate further if there was a significant difference within three clove extracts, three antibiotics, various concentrations, studied bacteria and interaction effect between them. P values <0.05 were considered as significant.

Results

The results reveal that studied three concentrations of clove extract as well as antibiotics have effective activity against all tested bacteria (Table-1). In clove extracts, the zone of inhibition was ranged from 0.00 to 12.00 mm. For 200 $mgml^{-1}$, it was ranged from 0.00 to 7.83 mm, 0.00 to 9.67 mm for 400 $mgml^{-1}$, and 7.33 to 12.00 mm for 600 $mgml^{-1}$. In three type of extracts, acetone gave the best results (10.00 to 12.00 mm) followed by methanol (7.50 to 9.83 mm) and ethanol (7.33 to 9.33 mm) at highest concentration (600 $mgml^{-1}$). In case of antibiotics, the zone of inhibition was ranged from 0.00 to 11.67 mm. For 0.02 $mgml^{-1}$ it was ranged from 0.00 to 8.67 mm, 0.00 to 9.67 mm for 0.04 $mgml^{-1}$, and 0.00 to 11.67 mm for 0.06 $mgml^{-1}$. In three type of antibiotics, ciprofloxacin gave best results (10.5 to 11.67 mm) followed by tetracycline (0.00 to 11.00 mm), while erythromycin did not show any activity. Statistical results of antibacterial activity of three clove extracts showed significant differences ($P<0.0001$) in efficacy among the bacterial strains (S), concentrations (C) of clove extracts (E), type of extracts as well as their interaction cases- S×C, S×E, C×E and S×C×E (Table 2).

Table 1. Antibacterial activities of three solvents extract of *S. aromaticum* L. and three antibiotics.

	Concentrations (mgml^{-1})		Zone of inhibition (mm)				
			S. typhimurium	*E. coli*	*S. dysenteriae*	*P. mirabilis*	*Y. enterocolitica*
Clove extracts	200	ET	0.00	0.00	0.00	0.00	0.00
		ME	0.00	0.00	0.00	0.00	0.00
		AC	7.33 ± 0.33	7.33 ± 0.33	7.83 ± 0.441	0.00	7.50 ± 0.29
	400	ET	7.00 ± 0.00	0.00	0.00	7.67 ± 0.33	0.00
		ME	8.33 ± 0.33	0.00	0.00	0.00	7.50 ± 0.50
		AC	9.33 ± 0.33	9.33 ± 0.33	9.67 ± 0.33	8.17 ± 0.44	9.33 ± 0.33
	600	ET	9.33 ± 0.33	7.50 ± 0.29	7.33 ± 0.33	9.33 ± 0.33	8.00 ± 0.58
		ME	9.83 ± 0.33	7.67 ± 0.66	7.50 ± 0.29	7.50 ± 0.29	9.33 ± 0.33
		AC	11.17 ± 0.17	12.00 ± 0.58	11.17 ± 0.44	10.00 ± 0.58	11.33 ± 0.33
Antibiotics	0.02	CP	8.67 ± 0.33	7.33 ± 0.33	7.33 ± 0.33	7.33 ± 0.33	7.17 ± 0.33
		TC	0.00	0.00	0.00	0.00	0.00
		ER	0.00	0.00	0.00	0.00	0.00
	0.04	CP	9.67 ± 0.33	9.00 ± 0.58	9.50 ± 0.29	8.33 ± 0.33	9.33 ± 0.33
		TC	0.00	8.67 ± 0.33	0.00	0.00	0.00
		ER	0.00	0.00	0.00	0.00	0.00
	0.06	CP	11.00 ± 0.58	11.67 ± 0.33	11.33 ± 0.33	10.50 ± 0.29	10.67± 0.33
		TC	0.00	11.00 ± 0.58	0.00	0.00	0.00
		ER	0.00	0.00	0.00	0.00	0.00

ET = Ethanol extract; ME = Methanol extract; AC = Acetone extract; CP = Ciprofloxacin; TC = Tetracycline; ER = Erythromycin. *Data were representing mean zone of inhibition (mm) ± SEM of three replicates.

According to the LSD test results (Table 3), means of strain, no significant differences were observed among *E. coli*, *S. dysenteriae* and *P. mirabilis*. But *S. typhimurium* and *Y. enterocolitica* were significantly different from others and between themselves. Highest mean value was found against *S. typhimurium* (6.926) and decreasing order of sensitivity of selected species of bacteria against three extracts was *Y. enterocolitica* (5.889) > *E. coli* (4.87) > *S. dysenteriae* (4.833) > *P. mirabilis* (4.741). In case of mean values of concentration, increasing the concentration level for extracts had a significant (P<0.05) inhibitory effect on all test bacteria. The inhibition area that found is larger as concentration of extracts is increased and highest for 600 mgml^{-1} (9.267) followed by 400 mgml^{-1} (5.089) and 200 mgml^{-1} (2.00). The mean value of extracts shown acetone (8.767) was significantly different from methanol (3.844) and ethanol (3.744), while no differences ware found between methanol and ethanol.

Table 2. Statistical results (ANOVA) of antibacterial activity of three clove extract.

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value	Prob.
Replication	2	0.104	0.052	0.1718	-
Strains (S)	4	96.94	24.23	81.79	0.00
Concentrations (C)	2	1196.99	598.50	2019.93	0.00
S×C	8	117.67	14.71	49.64	0.00
Extracts (E)	2	741.92	370.96	1251.98	0.00
S×E	8	204.70	25.59	86.36	0.00
C×E	4	71.06	17.77	59.96	0.00
S×C×E	16	173.70	10.88	36.70	0.00
Error	88	26.563	0.30	-	-
Total	134	2629.94	-	-	-

Like clove extracts, antibacterial activity of three antibiotics showed significant differences (P<0.0001) among the bacterial strains (S), concentrations (C) of antibiotics, type of antibiotics (A) as well as for interaction items- S×C, S×A, C×A and S×C×A (Table-4). Mean separation (Table-5) for antibacterial activity of antibiotics shows that the mean of strain *E. coli* (5.296) in the top and significantly different from

others. Rest of strains shows no differences among themselves. Here also concentrations of antibiotic were different from each other. All antibiotics were significantly different from each other and highest for ciprofloxacin (9.267) followed by tetracycline (1.311) and erythromycin (0.00).

Table 3. Analysis of mean data of the antibacterial activity of three clove extracts.

Variables	Growth inhibition diameter (mm)
Strains	
S. typhimurium	6.926 A
E. coli	4.87 C
S. dysenteriae	4.833 C
P. mirabilis	4.741 C
Y. enterocolitica	5.889 B
LSD	0.5148
Concentrations	
200 mgml^{-1}	2.00 C
400 mgml^{-1}	5.089 B
600 mgml^{-1}	9.267 A
LSD	0.3988
Extracts	
Ethanol	3.744 B
Methanol	3.844 B
Acetone	8.767 A
LSD	0.3988

Means followed by different letter(s) down the column are significantly different at $P<0.05$. Data values are means of three replicates.

Table 4. Statistical analysis of antibacterial activity of three antibiotics.

Source of variation	Degree of Freedom	Sum of squares	Mean Square	F Value	Prob.
Replication	2	0.181	0.091	0.5577	-
Strains (S)	4	107.57	26.89	166.91	0.00
Concentrations (C)	2	80.12	40.06	248.63	0.00
S×C	8	63.81	7.98	49.51	0.00
Antibiotics (A)	2	2263.22	1131.61	7023.77	0.00
S×A	8	207.28	25.91	160.81	0.00
C×A	4	48.83	12.21	75.77	0.00
S×C×A	16	102.36	6.40	39.71	0.00
Error	88	14.319	0.16	-	-
Total	134	2887.66	-	-	-

The MIC and MBC results of clove extracts are presented in Table 6. The results reveal that MIC values were ranged from 180 (*S. dysenteriae*) to 620 mgml^{-1} (*E. coli* and *S. dysenteriae*). For ethanol extract, it was ranged from 400 (*P. mirabilis*) to 620 mgml^{-1} (*E. coli* and *S. dysenteriae*), 400 (*S. typhimurium*) to 600 mgml^{-1} (*E. coli*, *S. dysenteriae* and *P. mirabilis*) for methanol, and 180 (*S. dysenteriae*) to 400 mgml^{-1} (*P. mirabilis*) for acetone.

Table 5. Analysis of mean data of the antibacterial activity of three antibiotics.

Variables	Growth inhibition diameter (mm)
Strains	
S. typhimurium	3.259 B
E. coli	5.296 A
S. dysenteria	3.13 B
P. mirabilis	2.907 B
Y. enterocolitica	3.037 B
LSD	0.3782
Concentrations	
0.02 mgml^{-1}	2.533 C
0.04 mgml^{-1}	3.633 B
0.06 mgml^{-1}	4.431 A
LSD	0.293
Antibiotics	
Ciprofloxacin	9.267 A
Tetracycline	1.311 B
Erythromycin	0.00 0 C
LSD	0.293

Means followed by different letter(s) down the column are significantly different at $P<0.05$. Data values are means of three replicates.

In three types of extract, acetone extract gave lowest MIC value (180 mgml^{-1}) against *S. dysenteriae* followed by methanol and ethanol (400 mgml^{-1}). In case of MBC values, it was ranged from 220 to 680 mgml^{-1} under the same strain (*S. dysenteriae*). For ethanol extract, it was ranged from 450 (*S. typhimurium* and *P. mirabilis*) to 680 mgml^{-1} (*S. dysenteriae*), 450 (*S. typhimurium*) to 650 mgml^{-1} (*E. coli*, *S. dysenteriae* and *P. mirabilis*) for methanol and 220 (*S. dysenteriae*) to 450 mgml^{-1} (*P. mirabilis*) for acetone. In three types of extracts, acetone extract gave lowest MBC value (220 mgml^{-1}) against *S. dysenteriae* followed by methanol and ethanol (450 mgml^{-1}).

Discussion

Although, the primary purpose of spices is to impart flavor and piquancy to food, the medicinal, antimicrobial and antioxidant properties of spices have also been exploited (Souza *et al.,* 2005). Cloves are antimutagenic (Miyazawa and Hisama, 2003), anti-

inflammatory (Kim *et al.,* 1998), antioxidant (Chaieb *et al.,* 2007a), antiulcerogenic (Bae *et al.,* 1998; Li *et al.,* 2005), antithrombotic (Srivastava and Malhotra, 1991) and antiparasitic (Yang *et al.,* 2003). On the basis of this information, antibacterial activities of clove extracts were evaluated for their antidiarrheal properties.

Table 6. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of three solvent extracts of *S. aromaticum* L. against studied bacterial strains.

	Extracts	Concentrations ($mgml^{-1}$)				
		S. typhimurium	*E. coli*	*S. dysenteriae*	*P. mirabilis*	*Y. enterocolitica*
MIC	ET	420	620	620	400	580
	ME	400	600	600	600	420
	AC	200	200	180	400	200
MBC	ET	450	650	680	450	620
	ME	450	650	650	650	480
	AC	250	250	220	450	250

ET = Ethanol extract; ME = Methanol extract; AC = Acetone extract

The antimicrobial activity has been attributed to the presence of some active constituents in the extracts. Clove contains a high eugenol (70-90%) content (de Guzman and Siemonsma, 1999) which is an antibacterial compound having wide spectra of antimicrobial effects against enterobacteria (Burt and Reinders, 2003; Nanasombat and Lohasupthawee, 2005; Chaieb *et al.,* 2007b). The results of this study exemplifies that clove extracts have potential source of antidiarrheal properties because extracts and their concentrations have significant influence on the growth of diarrhea causing bacteria and also it has superior antibacterial activity than antibiotics. Several investigators conducted related investigation and recommend clove extracts as a source of antibacterial agent (Nascimento *et al.,* 2000; Saeed and Tariq, 2008). It has also been reported that, clove oil potently inhibited the growth of different Gram negative bacteria (Saeed and Tariq, 2008; Lopez *et al.,* 2005). Clove extracts had a high activity against *E. coli* (12.00 mm) and previous studies have documented that *E. coli* are known to be multi-drug resistant bacteria (Saeed *et al.,* 2007; Singh *et al.,* 2002). The results were also in accordance with those reported by many investigators (Mandee *et al.,* 2003; Smith-Palmer *et al.,* 2001; Dorman and Deans, 2000; Hammer *et al.,* 1999; De *et al.,* 1999). All the tested bacteria, which were resistant to erythromycin and tetracycline, but significantly inhabited by clove extracts. Salman *et al.* (2008) also found comparable results. In this experiment, 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), microbial growth, exposure of micro- organisms to plant oil, the solubility of oil or oil components and the use and quantity of an emulsifier (Bansod and Rai, 2008). Increasing of the concentrations level of extracts had a significant ($P<0.05$) inhibitory effect on all studied bacteria. Similarly Tylor *et al.* (2001) reported that active compounds may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed and lack of activity can thus only be proven by using large dose (Farnsworth, 1993). Extracts prepared in acetone extract gave better activity than that of other extracts, and it could be better solubility of active components in acetone. It has been reported that different phytoconstituents have different degrees of solubility in different types of solvents depending on their polarity (El-mahmood and Ameh, 2007). This observation clearly indicates that the polarity of antimicrobial compounds make them more readily extracted by acetone solvent, and using organic solvent does not negatively affect their bioactivity against bacterial species (Kaushik and Goyel, 2008). Of all the bacteria, *S. typhimurium* was the most susceptible against all of the extracts and concentrations of clove while *P. mirabilis* was the most resistance bacteria. In

this study the low MIC and MBC values observed for *S. dysenteriae* 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). In all cases, three clove extracts consistently displayed superior potency when compared with antibiotics, while extracts are a mixture of various plant constituents and antibiotic is a refined and purified product (El-Mahmood and Doughari, 2008). Comparing among the three extracts with positive control, acetone extract was found most effective for antibacterial activity and the degree of antibacterial property of three extracts can be put in the following order: acetone > methanol > ethanol.

The results of this study revealed that although crude extracts of clove are not purified but their activity was very effective against all tested bacteria, and these extracts could be used as an effective antimicrobial agent, alternative to the use of antibiotics.

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