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Antibacterial Activity of *Allium Sativum, Syzygium Aromaticum,* and *Cinnamomum Zeylanicum* against Food Borne Pathogens *in Vitro*

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Abstract: *It is high time to search for novel antimicrobial agents as the bacterial drug resistance is increasing day by day. Spices have been believed to contain medicinal values from ancient time and posses less side effects in comparison to commercial drugs. Our study compares the antibacterial activity of ethanol extracts of Garlic (Allium sativum), Clove (Syzygium aromaticum), and Cinnamon (Cinnamomum zeylanicum) against Escherichia coli, Vibrio cholera, Staphylococcus aureus, Bacillus cereus, Salmonella sp, Shigella sp, Serratia sp, Acinetobacter sp, and Klebsiella sp. The primary antibacterial activity and minimum inhibitory concentration (MIC) were determined by agar well diffusion and broth dilution methods, respectively. Ethanol extracts of garlic, clove, and cinnamon showed broad-spectrum antibacterial activity against all the tested organisms, although the highest sensitivity was found from Staphylococcus aureus with all the three extracts. Garlic showed the highest zone of inhibition (ZOI) of 28 mm against S. aureus that is comparable to the effect of various commercially used antibiotics. However, garlic and clove showed the synergistic effect of 33 mm ZOI whereas they individually showed 28 and 26 mm ZOI, respectively, against S. aureus.*
Our result might be useful to apply the extracts as an alternative source of treatment for infections and other diseases. Their precise use can be useful in food preservation, as well, following detailed studies.
Keywords*: Antibacterial activity, Ethanol Extract, MIC, MBC and Synergistic effect*

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I Introduction

Food borne illness is a global issue because of failure in the management of good food safety practices[1]. According to world health organization, at least 2 billion people worldwide are getting ill annually due to consumption of unsafe food and that can be deadly as well[2]. The centers for disease control and prevention estimated that in every 6 persons at least 1 person become ill and thousands die annually in the United States[3, 4]. Though, the situation might be worse in the developing or underdeveloped countries.

From years after years, natural plant parts as spices have been used to enhance flavor, and day by day, their potentiality to possess medicinal properties including antimicrobial, antioxidant and anticancer have been discovered[5]. In addition, spices are also proved to utilize as preservatives by increasing the shelf life of foods[6]. Spices are getting immense interest due to their reduced side effects, addiction, availability and safety[7]. Moreover, spices with antimicrobial potential can replace the use of commercial antimicrobial agents as the drug resistance is increasing day by day due to their misuse[8, 9, 10]. World Health Organization adopted a policy to use more traditional medical practices for primary health care by the developing countries [11].

The aim of our experiment was to evaluate the antimicrobial potential of three spice extracts against nine bacterial pathogens. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were investigated to compare the antimicrobial potential of those three spices against nine food borne pathogens. Besides, we also performed antimicrobial activity of spices in combination to observe if they possess any synergistic effect.

II. Materials And Methods

Isolation and preparation of spice samples

Three most commonly used spices, namely, cinnamon (*Cinnamomum zeylanicum*), garlic (*Allium sativum*), clove (*Syzygium aromaticum*) were purchased from the supermarket at Banani in Dhaka Metropolitan City. The inner bark of cinnamon, fresh bulb of garlic, and aromatic flower bud of clove were collected in sterile zip-lock bags (Table 1). The spices were cleaned, washed with sterile distilled water, sliced, and dried in a tray in a hot air oven at 50˚C for 18-72 hours. Dried spices were kept in a sterile plastic bag unit in a refrigerator.

Media and Chemicals

Mueller Hinton Broth (MHB), Mueller Hinton Agar (MHA), Trypticase Soy Agar (TSA), Trypticase Soy Borth (TSB), Nutrient Broth (NB) and Nutrient Agar (NA) media were used. The antibiotics used were Gentamycin (GEN), Azithromycin (AZI), Neomycin (NEO), Tetracycline (TET), Streptomycin (STR), Rifampin (RIF), Ciprofloxacin (CIP), and Meropenem (MER). All chemicals used were of analytical-reagent grade.

Test microorganisms

The nine bacterial strains were tested for our study. Among them, *Escherichia coli* (ATCC 25922), *Vibrio cholera*e (ATCC 14035), *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (ATCC 14579), and *Salmonella typhi (ATCC 14028)* were collected from Dhaka University, Dhaka, Bangladesh. Other four strains including *Acinetobacter* sp, *Klebsiella* sp, *Shigella* sp, and *Serratia* sp were isolated from different food borne diseases in the Microbiology Department, Primeasia University, Dhaka, Bangladesh.

Preparation of inoculum

Bacterial strains were grown on TSA plates. Bacterial inoculums were prepared from overnight grown cultures in peptone water, and the turbidity was adjusted to 0.5 McFarland units (approximately, 10^8 CFU/ml for bacteria). A 0.5 McFarland standard was prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_20$), with 9.95 ml of 1% sulfuric acid (H_2SO_4) in a test tube with constant stirring[12].

Preparation of extracts

The clean and dried spices were minced in sterilized blender. Then, 10 gm of spices were soaked in 90 ml of 95% ethanol in sterilized Duran glass bottle and shaken at 250 rpm in a reciprocal shaker (WIS-10, Wisecube, Germany) at room temperature for one hour. The ethanol fraction was separated using sterilized cheesecloth and filtered through sterilized Whatman filter paper (No.03). The ethanol was evaporated using a dry oven (ED53, Binder, Germany) at 40˚C. The extract was weighed, and dissolved in ethanol to a concentration of 200 mg/ml, and stored at refrigerator in a sterile vial for further experiments.

Antibacterial sensitivity testing

The antibacterial activity was carried out by agar well diffusion method. The selected bacterial strains were inoculated into 10 ml of sterile TSB and incubated at 37 ºC for overnight. The TSB cultures were inoculated with sterile cotton swab on the surface of sterile MHA plates. Following inoculation, agar wells of 8 mm in diameter, 4 mm deep and about 2 cm apart were punched in the MHA plate with a sterile cork borer. Then, 50 and 100 µl of those spice extracts were poured into labeled wells. The plates were allowed to stand for 3 hours for diffusion to take place before incubated at 37°C for 18-24 hours as described previously[13,14,15]. The antibacterial activity of commercial drugs was performed by disk diffusion method[16]. Subsequently, antibiotic discs were aseptically placed over the seeded MHA plate after inoculation, sufficiently separated from each other to avoid overlapping zone of inhibition. Following incubation under specified conditions, the results were evaluated as the diameter of the zone of inhibition (ZOI) in mm, and recorder. When microorganisms showing a clear zone of more than 12 mm, between 12 and 16 mm, and greater than 16 mm, the extracts were considered to be active, moderately active and highly active, respectively[17]. Each experiment was performed in triplicate and mean values were taken.

Determination of MIC and MBC

The MICs of the crude extracts were determined using the doubling dilution method[18]. In brief, reconstituted ethanol extract at a concentration of 512 µl/ml was prepared in NB and mixed well. Following mixing, 1 ml of this extract concentration was transferred to another test tube containing 1ml NB, and this dilution continued to give extract concentrations of 512, 256, 128, 64, 32, 16, and 8 µl/ml. Then, 100 µl of an 18-hour culture of bacteria was inoculated into each of the test tubes, and the contents were thoroughly mixed. The tubes were incubated at 37ºC for 24 hours. Test tubes containing 1 ml of different concentrations of extracts and 1 ml of NB without culture were considered as controls. The test tube with the lowest concentration of the extract that did not show any detectable growth was taken as the MIC. Tubes showing no growth for the MIC was sub-cultured on the NA plate without extracts and incubated at 37°C for 24 hours. The lowest concentration of the extracts yielding no growth on the NA plate was recorded as the MBC.

Synergistic effect of extracts

Following determination of the MIC and MBC, the synergistic effect of the extracts in combination was tested. Here, the combination of 1/2 × MIC of the extracts were applied. The rest of the procedure was followed by agar well diffusion method as described previously.

III. Result

Antibacterial activity of spices

In this study, the antibacterial activity of three spice extracts was detected against nine different food borne microorganisms (Table 2). Following preparation of spice extracts, 100 µl of extracts were tested for their antibacterial activity using agar well diffusion method by the diameter of ZOI.

Table No 1: Properties of spices

Name of spices	Scientific name	Bengali/local name	Part of use	Genus	Family
Garlic	***Allium sativum***	Rasun	Bulbs	*Allium*	Amaryllidaceae
Clove	***Syzygium aromaticum***	Labaṅga	Flower Buds	*Syzygium*	Myrtaceae
Cinnamon	***Cinnamomum zeylanicum***	Daarchini	Inner Bark	*Cinnamomum*	Lauraceae

All the spices showed various degrees of ZOI against tested organisms. The diameter of ZOI for the bacterial strains obtained against garlic, clove, and cinnamon were 12 to 28, 11 to 26 and 14 to 22, respectively. Among the tested organisms, *S. aureus* showed the highest sensitivity against all the spices, whereas other organisms showed heterogeneous degrees of sensitivity against the spices. Garlic showed the widest ZOI by *S. aureus* with a diameter of 28 mm, followed by *Shigella* sp. with a diameter of 23 mm and *Acinetobacter* sp. with a diameter of 21 mm. Clove showed the highest ZOI by *S. aureus* with a diameter of 26 mm, followed by *Klebsiella* sp. and *B. cereus* with a similar diameter of 20 mm. Besides, cinnamon induced the highest 22 mm ZOI by S. *aureus*, followed by *Serratia* sp. with a diameter of 20 mm.

The antibacterial activity of the three spices was compared with eight commercially available antibiotics using disk diffusion method as shown in Table 3. The highest diameter of ZOI (28 mm) was found with Rifampin against *S. aureus*. Whereas, Ciprofloxacin showed the widest ZOI for the rest of the tested organisms. Moreover, our tested spice extracts showed convincing antibacterial potential while compared to commercial antibiotics.

Table No 2: Antibacterial activity of garlic, clove and cinnamon against food borne bacterial pathogens

Name of microorganisms	ZOI in mm		
	Garlic	Clove	Cinnamon
S. aureus	28	26	22
V. cholerae	15	14	15
***Klebsiella* sp.**	18	20	15
E. coli	18	15	18
***Shigella* sp.**	23	11	14
S. typhi	12	15	18
B. cereus	17	20	18
***Acinetobacter* sp.**	21	19	15
***Serratia* sp.**	19	16	20

Table No 3: Antibacterial activity of commercial drugs

Name of microorganisms	ZOI in mm							
	GN10	AZM15	RA5	N30	T30	S10	CIP5	MRP 10
S. aureus	18	22	28	18	24	0	24.3	0
***Serratia* sp.**	0	0	8	9	16	10	25	7.6
E. coli	14	0	0	13	0	8	0	7
V. cholerae	13	17	18	16	14	15	18.3	7
***Klebsilla* sp.**	17	0	0	0	0	0	24	7
***Shigella* sp.**	14	9	0	6	8	15	25.3	8
S. typhi	15	10	0	13	0	15	20	8.6
B. cereus	0	8	11	0	16	0	0	7.3
***Acinetobacter* sp.**	20	12	14	16	0	15	22	18

Determination of MIC and MBC

The MIC and MBC of garlic, clove and cinnamon extracts against tested bacterial strains are presented in Table 3. All the tested extracts showed concentration-dependent growth inhibition of the tested organisms. Garlic was the most effective spice with the MIC and MBC values ranged from 32 to 256 µl/ml and 64 to 512 µl/ml, respectively. The lowest MIC and MBC values of garlic extracts were observed against *Shigella* sp. Whereas, the MIC and MBC values of clove were ranged from 64 to 512 µl/ml and 128 to 512 µl/ml, respectively and of cinnamon were ranged from 64 to 256 µl/ml and 128 to 512 µl/ml, respectively. In general, the extracts showed MIC at 64 µl/ml and MBC at 128 µl/ml against most of the tested organisms.

Table No 4: MIC and MBC of garlic, clove and cinnamon against food borne bacterial pathogens

Name of microorganisms	Name of Spices					
	Garlic		Clove		Cinnamon	
	MIC (µl/ml)	MBC (µl/ml)	MIC (µl/ml)	MBC (µl/ml)	MIC (µl/ml)	MBC (µl/ml)
S. aureus	64	128	64	128	64	128
Serratia sp.	64	128	128	256	256	512
E. coli	64	128	512	512	128	256
V. cholerae	64	128	128	256	128	256
Klebsiella sp.	64	128	64	128	64	128
Shigella sp.	32	64	64	128	64	128
S. typhi	256	512	128	256	64	128
B. cereus	64	128	128	256	64	128
Acinetobacter sp.	64	128	64	128	128	256

Synergistic Effect

A drug that works at less concentration is always preferable. Here, we performed agar well diffusion method to detect the synergistic effect of every two spices at half the MIC against all the selected microorganisms. Our data showed that clove and cinnamon together produced a broader ZOI (25.3 mm) whether they individually showed 15 and 18 mm ZOI against *E. coli*. Moreover, clove and cinnamon showed a better effect against *Vibrio* sp. when used in combination. Furthermore, garlic and clove together also showed broader ZOI in comparison with their individual effect against *S. aureus*, *Vibrio*, and *Acinetobacter*. Here, garlic and clove in combination showed 33 mm ZOI whereas, they separately showed 28 and 26 mm ZOI against *S. aureus*.

Table No 5: Synergistic effect of garlic, clove and cinnamon in combinations against food borne bacterial pathogens

Name of microorganisms	Sample	Synergistic effect (ZOI in mm)
E. coli	Clove + Cinnamon	25.3
	Cinnamon + Garlic	19
	Garlic + Clove	17
S. aureus	Clove + Cinnamon	27
	Cinnamon + Garlic	29
	Garlic + Clove	33
S. typhi	Clove + Cinnamon	13
	Cinnamon + Garlic	14.3
	Garlic + Clove	17.3
V. cholerae	Clove + Cinnamon	24.6
	Cinnamon + Garlic	15.3
	Garlic + Clove	21.3
B. cereus	Clove + Cinnamon	23
	Cinnamon + Garlic	17
	Garlic + Clove	20
***Shigella* sp.**	Clove + Cinnamon	19
	Cinnamon + Garlic	16.5
	Garlic + Clove	17.5
***Serratia* sp.**	Clove + Cinnamon	16
	Cinnamon + Garlic	20
	Garlic + Clove	15
***Acinetobacter* sp.**	Clove + Cinnamon	18
	Cinnamon + Garlic	20
	Garlic + Clove	24
***Klebsiella* sp.**	Clove + Cinnamon	14.5
	Cinnamon + Garlic	16
	Garlic +Clove	15

IV. Discussion

All the tested spice extracts showed satisfactory antimicrobial activity against most of the microorganisms. Our study showed that the ethanol extract of garlic was highly active against *S. aureus*, *Shigella* sp, and *Acinetobacter* sp, whereas the concentration-dependent inhibitory activity of garlic against *S. aureus* was reported previously with the highest activity shown at 80 to 100 mg/ml concentrations[19]. In another study, garlic showed the highest inhibitory activity against *E. coli* among gram-negative respiratory tract isolates tested[20]. All the three spice extracts showed the maximum activity against *S. aureus*. In case of clove extract,

highest sensitivity was observed with *S. aureus*, followed by *B. cereus* and *Klebsiella* sp. that correlate with the previous report where distilled water and petroleum ether extracts of clove also showed highest inhibitory action against *S. aureus*[21]. Our cinnamon extract showed the highest inhibitory action against *S. aureus* followed by *Serratia* sp., *E. coli*, *S. typhi*, and *B. cereus*. However, in another study, Cinnamon extract showed the highest ZOI against *Bacillus cereus*, followed by *S. aureus*[22]. All the extracts showed great potential to act as antimicrobial agents when we compare with the result of eight very commonly used antibiotics. We also performed MIC and MBC for those extracts against nine microorganisms. Garlic at very low concentrations showed MIC against most of the tested microorganisms when we compare with clove and cinnamon.

We also detected either those extracts showed any synergy when added in combination. Interestingly, we got some promising data for some combinations. Clove and cinnamon in combination showed 25.3 mm ZOI, while they separately showed 15 and 18 mm ZOI against *E. coli*, respectively. In addition, garlic and clove also showed 33 mm ZOI in combination whether they separately showed 28 and 26 mm ZOI against *S. aureus*, respectively. These combinations of extracts surpassed the ZOI values in comparison with those extracts when treated separately. Those combinations also showed greater antibacterial activity in comparison with any commercial antibiotic tested. It was reported that mixtures of spices help to stabilize color and smell of fresh portioned pork meat by inhibiting the growth of meat spoiling bacteria, namely, *B. subtilis*, *Enterococcus* sp, *Staphylococcus* spp, *E. coli* K 12 and *Pseudomonas fluorescence*[23]. Therefore, combinations of spices and other antimicrobial agents may increase food shelf life by destroying food spoilage organisms and may use as alternative drugs to treat mild sickness instead of using commercial drugs as well.

Uncontrolled use of antibiotics in treatment and chemical antimicrobial preservatives in food preservation are the major causes of emerging drug resistance day by day. Fifty years of misuse or uncontrolled use of antimicrobials leading to multi-drug resistant microorganisms and ecological imbalance that is alarming for the very near future.

V. Conclusion

It is concluded from the present study that all the three spice extracts have promising antibacterial activity. Interestingly, some combinations of spices induced the inhibitory activity of specific microorganisms. Although researcher kept an eye on the antimicrobial properties of spices from the recent past, little is known about the defined mechanism of those spices, their effect on individual pathogens, synergy when used in combinations with other spices or antibiotics. Hence, detailed studies are required and at the same time, continuous screening of new antimicrobial agents is a necessity to overcome the drug resistance issue.

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