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Original Article

Microbial Contamination and Detection of Antibacterial Activity of *Syzygium aromaticum* against Food Borne Pathogens

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This study was undertaken to find out the presence of contaminating microorganisms in commonly available *Syzygium aromaticum* samples collected from different areas of Bangladesh and also to evaluate the antibacterial traits of these *Syzygium aromaticum* samples against food born pathogens. Total viable bacterial count (TVBC) was determined on nutrient agar and for the isolation of specific microorganisms different selective media were used. Crude, ethanol, methanol, hot water and cold water extracts of the samples were prepared for analysing their antibacterial activity using the agar well diffusion method. Furthermore, the minimum inhibitory concentration (MIC) of the crude extracts was determined. TVBC was found between 10^4 to 10^6 cfu/g. None of these samples showed the presence of fungus. *Staphylococcus* spp. was present almost in all the samples between 10^4 to 10^6 cfu/g while *Bacillus* spp. was noticed only in one sample. *In vitro* antibacterial activity of the crude, methanolic and ethanolic extracts of the samples was found to be effective mostly against *Escherichia coli, Klebsiella* spp., *Listeria* spp., *Pseudomonas* spp. and *Bacillus* spp. On the contrary, hot water extracts of only two samples showed antibacterial property against *Pseudomonas* spp., *Listeria* spp. and *Klebsiella* spp. MIC was confirmed by using 96 well plate methods and the minimum inhibitory concentration was between 11.75 to 94 mg/ml.

Introduction

For many centuries spices are used by all countries to augment flavour and aroma in different types of food and in treatment of clinical ailments[1]. Antibiotics supply the main root for the treatment of microbial infections[2]. Microorganisms have become resistant to many antibiotics due to increased use of drugs. So, it has become essential to find out new antimicrobial agents[3,4].

The most common bacteria causing food-borne illness are *Escherichia coli*, *Staphylococcus*, *Salmonella* spp., *Listeria monocytogenes*, *Clostridium botulinum*, *Vibrio vulnificus*, *Vibrio parahaemolyticus* and others[5,6]. *Syzygium aromaticum* may be contaminated because of the surroundings under which they were cultivated and harvested. Contaminated *S. aromaticum* have been reported for the cause of certain food-borne illnesses and spoilage[7]. Therefore, *S. aromaticum* sometime pose health trouble because they are often added to foods without supplementary processing or are eaten uncooked[8].

Cloves of *Syzygium aromaticum* are the pungent dried flower buds of a tree in the family Myrtaceae. Cloves are local to Indonesia and used as a spice in cuisines worldwide[9]. It is also used as a carminative, rubefacient and serves as a preservative in herbal recipes, signifying possible antimicrobial property[5,10,11]. *S. aromaticum* contain compounds like gallotannins, triterpenes, flavonoids, and phenolic acids[12,13].

In Bangladesh, although a lot of works has been conducted based on herbal plants and medicines, there is a little information on the sources of contamination and antibacterial activity of cloves. Based on this consideration the current study was designed to detect the microbial contamination and detection of antibacterial activity of *Syzygium aromaticum* collected from various parts of Bangladesh.

Methods & materials

Study area, sampling and sample processing

Ten *Syzygium aromaticum* samples were randomly collected from Comilla, Reazuddin Bazar (Chittagong), Pahartali (Chittagong), Shantinagor (Dhaka), Gazipur, Ashulia, Tongi, Uttara (Dhaka), Rajshahi and Netrokona during August 2015-November 2015 following the standard protocol[15,16]. For the detection of contaminating bacteria and fungi, 10 g of each sample was homogenized with 90 mL normal sterile saline and serially diluted up to 10^{-5}.

Microbiological analysis

For each of the samples, 0.1 mL from the dilution 10^{-2} and 10^{-5} were introduced on to the Nutrient agar (Hi-Media Laboratories Pvt. Ltd., India) and sabouraud dextrose agar (Hi-Media Laboratories Pvt. Ltd., India) for the enumeration of total viable bacteria and fungi, respectively. Consequently, different selective

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media such as MacConkey agar (Hi-Media Laboratories Pvt. Ltd., India), Mannitol salt agar (Merck Specialities Pvt. Ltd, Mumbai, India), Cetrimide agar (Hi-Media Laboratories Pvt. Ltd., India), Starch agar (manually produced by using peptone, beef extract, bacterial agar and starch), Salmonella Shigella agar (SS Agar), and Thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Hi-Media Laboratories Pvt. Ltd., India) were used for the detection of coliforms, *Staphylococcus* spp. *Pseudomonas* spp, *Bacillus* spp., *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. respectively[16]. Alkaline Peptone Water (APW) was used to enrich *Vibrio* spp. and Selenite Cystine Broth (SCB) was used to enrich *Salmonella* spp. and *Shigella* spp. for 3 hours. All the inoculated plates were incubated at 37 °C for 24 hours except Sabouraud Dextrose agar plates, which were incubated at 25 °C for 48 hours[17].

Crude extraction

3.33 g of dried and blended sample were soaked in 10 mL of normal sterile saline (maintaining the ration of 25 g of sample with 75 mL of normal saline)[17].

Hot water extraction

5 g Dried sample were soaked with 45 mL of sterile distilled water (maintaining the ration of 10 g sample with 90 mL of the water) and boiled at 100 °C for 10 minutes in Durham's bottle (Schott Duran, Germany) (i.e., distilled water extract) and kept in shaking water bath (Daihan Scientific Co., Ltd, Korea, Model No-WSB-30) at 130 rpm/min for 24 h at 20 °C. Samples were aseptically filtered through sterile Whatman No 1 filter paper (Hangzhou Xinhua Paper Industry Co., Ltd., Hangzhou, China). Then the liquid portion was collected[18].

Solvent extraction

Subsequently, 5 g of the dried clove powder of each sample were added with 45 mL of ethanol and methanol(maintaining the ratio of 10 g sample with 90 mL ethanol and methanol) in Durham's bottle and were kept in shaking water bath (WSB-30, Korea) at 130 rpm/min for 24 hours at 20 °C. After filtration the fluid section was collected[19,20,21].

Antimicrobial assay

Modified agar well diffusion method was followed using Mueller-Hinton agar (MHA) plates (Oxoid Ltd., Basingstoke, Hampshire, England). The suspension of *Escherichia coli* (*E. coli*), *Pseudomonas* spp., *Salmonella* spp., *Listeria* spp., *Vibrio* spp., *Klebsiella* spp., *Staphylococcus* spp. and *Bacillus* spp. were introduced on to the wells (8 mm) of the MHA media. Then 100 µl of the samples (crude extract, Distilled water extract, autoclaved hot water extract, ethanol extract, methanol extract) at a concentration of 11.1 mg/ml were introduced. Besides absolute ethanol (Merck Specialities Pvt. Ltd, Germany), methanol (Merck Specialities Pvt. Ltd, Germany) and normal sterile saline as negative control were applied. Antibiotic disc of gentamicin 10 µg (Oxoid Ltd., Basingstoke, Hampshire, England) was used as a positive control. Plates were incubated at 37 °C for 12-18 h, and were examined for the determination of zone of inhibitions (mm)[17,22,23].

Determination of Minimal Inhibitory Concentration (MIC)

For the detection of antibacterial activity of clove, the minimum inhibitory concentration (MIC) or broth microdilution assay was demonastrated[4,24]. An aliquot of 10 µL of each bacterial culture (ovenight growth, ~12 hours) was inoculated into the appropriately labeled sterile tubes containing Mueller Hinton broth (MHB) (Oxoid Ltd, England) at the turbidity adjusted with 0.5 McFarland standard. Afetrward different volumes of the samples (16 µL, 32 µL, 64 µL, 128 µL, and 256 µL) were introduced to make a total volume of 300 µL. After incubation at 37 ºC for 24 hours all the tubes were observed and recorded the lowest concentration (mg/mL) of each sample in which the bacterial cell was found to be retarded and considered as the MIC value[2,15,24].

Results

Microbial Analysis of Syzygium aromaticum (clove) samples:

In the present study, TVBC was observed within the range of 3×10^4 to 5.3×10^7 cfu/g. Among the specific isolates, coliform, *Salmonella, Vibrio, Pseudomonas* and fungi were not detected in any of the clove samples. *Staphylococcus* spp. was the most prevalent bacteria, found almost in all samples between 1×10^4 to 1.08×10^6 cfu/g (Table 1). *Bacillus* spp. ($1.83\times10^{7)}$) was only found in sample collected from Reazuddin Bazar.

Table 1. *Microbiological analysis of Syzygium aromaticum (clove) samples*

Sample No.	Sample	TVBC	*Staphylococcas*spp.
1	Comilla	9.0×10^5	4×10^5
2	Reazuddin bazar(Chittagong)	5.3×10^7	0
3	Pahartali (Chittagong)	3×10^4	0
4	Shantinagor (Dhaka)	1×10^6	0
5	Gazipur	2.62×10^6	1×10^4
6	Ashulia	1.01×10^6	1.0×10^5
7	Tongi	2×10^6	2.4×10^5
8	Uttara (Dhaka)	2×10^6	1.08×10^6
9	Rajshahi	4×10^6	1.1×10^5
10	Netrokona	5×10^6	3×10^5

Maximum limit (cfu/g) of microorganisms in spices: (According to ICMSF: 1998)[25]

- total viable bacterial count (TVBC): 10^6 cfu/g
- fungi: 10^4 cfu/g
- Coliforms and *E. coli*: 10^3 cfu/g

Antibacterial activity of Syzygium aromaticum (Clove) extracts (crude Extraction):

Crude extracts of sample nos. 1, 9 and 10 showed antibacterial activity against *E. coli*. No activity of the crude extracts was found against *Salmonella* spp. and *Pseudomonas* spp. Similar types of zone of inhibition was observed for *Listeria* spp., *Vibrio* spp. and *Staphylococcus* spp. whereas variable result was noticed against *Bacillus* spp. (Table 2).

Table 2. *Antibacterial activity of crude extraction: zone of inhibition (mm)*

Sample No.	*E. coli*	*Staphylococcus* spp.	*Bacillus* spp.	*Vibrio* spp.	*Klebsiella* spp.	*Listeria* spp.
1. Comilla	10	13	0	12	18	13
2. Reazuddin bazar(Chittagong)	0	12	0	11	17	12
3. Pahartali (Chittagong)	0	16	11	12	18	10
4. Shantinagor (Dhaka)	0	13	12	12	17	10
5. Gazipur	0	12	14	11	15	10
6. Ashulia	0	11	13	11	0	13
7. Tongi	0	0	11	0	0	10
8. Uttara (Dhaka)	0	12	0	0	14	12
9. Rajshahi	10	13	0	0	18	12
10. Netrokona	10	0	0	0	17	11
11. Positive control (Gentamicin)	17	15	14	14	18	17
12. Negative Control (Normal saline)	0	0	0	0	0	0

The results of antibacterial activities (Ethanol, Methanol and Hot water extraction):

By analyzing the results, mostly *Syzygium aromaticum* has antibacterial activity against *E. coli, Klebsiella* spp. *Listeria* spp. and *Pseudomonas* spp, *Bacillus* spp. in comparison to the clear zone of the positive control Gentamicin (10 ¼g) as standard (≥15mm)[26]. Antibacterial activity was found for three samples against *Vibrio* spp. and little was shown against *Staphylococcus* spp. and *Salmonella* spp. (Table 3).

Table 3. *Bacterial isolates susceptible against various extracts of Syzygium aromaticum Collected from different areas of Bangladesh*

Sample No.	Sample	Bacterial isolates susceptible (zone of inhibition in mm) against various extracts of *Syzygium aromaticum*		
		Ethanol extraction	**Methanol extraction**	**Hot water extraction**
1	Comilla	*E. coli* (21mm), *Staphylococcus* spp. (20mm), *Vibrio* spp. (18mm), *Pseudomonas* spp. (17mm), *Listeria* spp. (18mm)	*E. coli* (20mm), *Klebsiella* spp. (15mm), *Staphylococcus* spp. (18mm), *Vibrio* spp. (20mm), *Pseudomonas* spp .(18mm), *Listeria* spp. (19mm)	
2	Chittagong (Reazuddin bazar)	*E. coli* (18mm), *Pseudomonas* spp. (16mm)	*Klebsiella* spp. (20mm), *Listeria* spp. (16mm)	
3	Chittagong (pahartali):	*Klebsiella* spp. (23mm), *Bacillus* spp. (15mm), *Pseudomonas* spp .(19mm)	*Klebsiella* spp. (23mm), *Pseudomonas* spp.(19mm), *Listeria* spp. (20mm)	*Klebsiella* spp. (17mm), *Listeria* spp. (17mm)
4	Dhaka (Shantinagar)		*E. coli* (15mm)	
5	Gazipur	*E. coli* (19mm), *Listeria* spp.(15mm)	*E. coli* (19mm)	
6	Ashulia (Dhaka)	*E. coli* (19mm), *Bacillus* spp. (15mm)		
7	Tongi	*Vibrio* spp. (15mm)	*E. coli* (16mm), *Vibrio* spp. (15mm)	
8	Uttara (Dhaka)	*E. coli* (24mm). *Bacillus* spp. (17mm), *Vibrio* spp. (16mm), *Pseudomonas* spp. (22mm), *Listeria* spp. (17mm)	*E.coli* (23mm), *Bacillus* spp. (15mm) , *Pseudomonas* spp. (23mm), *Listeria* spp. (21mm)	*Pseudomonas* spp. (17mm)
9	Rajshahi	*E.coli* (22mm), *Klebsiella* spp. (19mm), *Listeria* spp. (19mm)	*Bacillus* spp. (18mm), *Staphylococcus* spp. (16mm)., *Salmonella* spp. (15mm), *Vibrio* spp. (20mm), *Listeria* spp. (25mm)	
10	Netrokona	*E.coli* (15mm), *Klebsiella* spp. (15mm), *Listeria* spp. (20mm)	*E.coli* (16mm), *(Klebsiella* spp. (18mm), *Bacillus* spp. (15mm), *Listeria* spp. (20mm)	

Table 4. *Determination of the minimum inhibitory concentration of the crude extracts (mg/ml)*

Sample	*Klebshiella* spp.	*Vibrio* spp	*Pseudomona s*spp.	*Staphylococcus* spp.	*Bacillus* spp.	*E. coli*	*Salmonella* spp.	*Listeria* spp.
Sample-1	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5
Sample-2	23.5	23.5	23.5	23.5	23.5	23.5	94	23.5
Sample-3	23.5	23.5	23.5	23.5	23.5	23.5	94	23.5
Sample-4	23.5	23.5	23.5	23.5	11.75	11.75	23.5	23.5
Sample-5	47	47	47	23.5	94	23.5	23.5	23.5
Sample-6	94	94	94	94	94	94	94	94
Sample-7	94	47	47	47	47	47	47	47
Sample-8	94	94	94	94	47	47	47	47
Sample-9	47	47	47	47	94	94	94	94
Sample-10	94	47	47	94	94	94	94	94

In each experiment, both the positive control Gentamicin (10µg) and the negative control of normal saline, ethanol and methanol were maintained. In all the cases, Gentamicin produced zone of inhibition around 15-18 mm against all the laboratory isolates and the negative controls didn't show zone of inhibition at all.

Minimum Inhibitory Concentration (MIC) assay of the crude extracts of the samples:

In this study, the MIC for the crude extract of various samples was determined. Unlike the results obtained from agar well diffusion method of the crude extracts of samples, the MIC study also revealed the inhibition of the growth for all the eight laboratory isolates. Interestingly MIC for the sample nos. 1-4 showed the growth retardation on an average of 23.5 mg/ml. On the other hand, for sample nos. 5-10 minimum inhibitory concentrations obtained was between of 47 to 94 mg/ml. Noticeably, *E. coli* and *Bacillus* spp. was inhibited at the concentration of 11.75 mg/ml by the sample no. 4.

Discussions

Medicinal plants play a vital role for the development of new drugs. Besides production of synthetic drugs, the biopharma industries in Bangladesh like Square Pharma and others are producing herbal medicines. During harvesting *Syzygium aromaticum* may come in contact with various types of microorganisms which may cause certain food-borne illnesses and spoilage[7]. Therefore current study was designed for analysing the microbiological contamination of *Syzygium aromaticum* (clove) samples collected from various areas of Bangladesh and also to determine their antibacterial activity against eight food borne pathogens.

Previous studies on the microbiology of spices have demonstrated contamination of microorganisms, including total heterotrophs, *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella* and toxigenic moulds[7] (ref). From the Table 1 it was observed that a substantial amount of total viable bacteria were present in almost all the samples. *Staphylococcus* spp. were present in all the samples. As it is known that *Staphylococcus* spp. is a normal flora of skin, the sample was possibly contaminated from skin flora (Table 1). Sample no. 2 contained *Bacillus* spp. One of the major reasons for this may be due to the contact of the sample with soil (Table 1)[27]. Sometime unprocessed clove is consumed as mouth freshener so it should not contain the microorganisms. This finding was not correlated with the findings of Parveen *et al*., (2014)[28] who beside detecting TVBC also found the presence of yeast, mould and coliform.

Ethanolic extract of various samples gave clear zone of inhibition against most of the laboratory isolates. The clear zone was better for mainly *Listeria* spp*., E. coli, Klebsiella* spp*. and Bacillus* spp. In case of methanolic extract better results were recorded against *E. coli, Listeria* spp*., Pseudomonas* spp. and *Klebsiella* spp. On the other hand *Staphylococcus* spp. and *Vibrio* spp. were found to be the most resistant isolate against both the methanolic and ethanolic extracts of clove samples[29]. In case of methanolic extraction better clear zones were establish than ethanol extraction. From Result from Pandey *et al*., (2011)[3] showed that extraction with ethanol against *Staphylococcus aureus* gave 20 mm inhibition zone and *Pseudomonas aeruginosa* give 18 mm zone of inhibition but no zone of inhibition was found against *E. coli* for both ethanol and methanol extraction.

As there was significant antibacterial activity found in agar diffusion assay, the minimum inhibitory concentration (MIC) of the clove extract for all the organisms were determined. The lowest MIC (11.75 mg/ml) was achieved for the sample no 4 and the maximum (94 mg/ml) concentration was found for sample nos. 4, 6, 8, 9 and 10. Interestingly all the tested laboratory isolates were found to be sensitive within this range. There hasn't been much previous report on MIC regarding the clove samples, although there are some studies on herbal medicine and related products. In a study conducted by Sharmin *et al*., (2014)[24] on herbal medicine showed the MIC was 10 mg/ml for the isolates which was pretty much similar to that of the lowest MIC (11 mg/ml) recorded in this study.

Conclusions

In vitro studies have revealed *Syzygium aromaticum* to have bacteriostatic, bactericidal property. The result of the antibacterial

traits of the samples reflected in this study strongly indicates the huge potentiality of *Syzygium aromaticum* (cloves) as a good candidate to be used as a medicinal plant to treat various food borne diseases caused by the pathogenic microorganisms. However further studies are needed to better estimate the accurate efficiency of the extracts as the antimicrobial agent. Besides as *Syzygium aromaticum* (cloves) are sometimes taken raw for various purposes, better hygienic approaches should be taken both at the consumer and the producer level to avoid any food borne infection.

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