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Evaluation of three commercial spices against pathogenic bacteria of traditional sweetmeat- rossomalai

Most. Ferdousi Begum*, Md. Abdul Qayum Sarker, Md. Mozammel Hoque, Md. Showkat Hossain, M. Firoz Alam

Department of Botany, Rajshahi University, Rajshahi 6205, Bangladesh

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Abstract

The antibacterial activity of three spices extracts namely *Syzygium aromaticum*, *Cuminum cyminum* and *Foeniculum vulgare* were assessed against seven pathogenic bacteria such as *Escherichia coli*, *Salmonella* sp., *Bacillus cereus*, *Klebsiella* sp., *Streptococcus* sp., *Staphylococcus aureus* and *Staphylococcus* sp. of Rossomalai. Among the spices, ethanol extract of *S. aromaticum* showed highest inhibition zone (15.8 mm) against *E. coli*. On the other hand, aqueous extract of *F. vulgare* seed demonstrated least activity against *S. aureus*. MIC and MBC value ranges from 25 to 200 mg ml^{-1} and 50 to 225 mg ml^{-1}, respectively. The lowest MIC and MBC values were recorded against *E. coli* for ethanol extract of *S. aromaticum* inflorescence. On the basis of antimicrobial spectra *S. aromaticum* can be considered as an effective antimicrobial agent that can be used as a food preservative in commercial purpose.

* **Corresponding Author:** Most. Ferdousi Begum ✉ ferdrita@yahoo.com

Introduction

Rossomalai is a traditional chhana based sweetmeat in Bangladesh. It is very nutritious on account of its fairly high protein, fat contain minerals, especially calcium and phosphorous and also few soluble vitamins particularly vitamin A and D (Alam *et al*., 2002) which are suitable for growth of microorganism. Recent U.S estimates indicate that about 76 million illnesses are attributed to food-borne disease and among them 30% are caused by bacteria, 3% by parasites, and 76% by viruses (Mead *et al*., 1999). Foods are the primary sources of many pathogenic microorganisms like *E. coli. Staphylococcus aureus, Clostridium botulinum, Listeria monocytogenes, Salmonella* etc. These organisms commonly carried by the nose and the skin can easily be transferred to food through handling, which causes infectious diseases like nausea, vomiting, or fever. Incidence of food borne diseases is common in Bangladesh where public health and sanitation facilities are inadequate (Kabirullah, 2006). In developed countries food-borne pathogens are responsible for millions of cases of infectious gastrointestinal diseases each year, costing billions of dollars in medical care and lost productivity. Recently there has been increasing interest in discovering new natural antibacterial (Sagdic *et al*., 2003a) to control and treatment of various infectious diseases as chemically synthesized drugs have undesirable side effects. The growing concern about food safety has lead to the development of natural antimicrobials to control food borne pathogens. Some spices are commonly used as natural antimicrobial agents in foods. Addition of spices in foods not only imparts flavor and pungent stimuli but also provides antimicrobial property (Nevas *et al*., 2004). In herbal medicine, cumin seed are used as galactogogue, stimulant, carminative, stomachic, and antispasmodic. Moreover, cumin oil shows a high antifungal activity against various pathogenic fungi and effective high antibacterial activity (Li and Jiang, 2004). Syrup is made from fennel to treat babies with colic or painful teething and long term ingestion (Türkyilmaz *et al*., 2008). Although some researchers have studied the antibacterial activity of spices against several species of bacteria, such as *E. coli, Salmonella*, *Shigella*, *L. monocytogenes* etc. and few serotypes of *Salmonella* i.e., *S. typhimurum* (Elgayyar *et al*., 2001), *S. enteritidis* (Tassou *et al*., 1995) and *S. anatum* (Swetwieathana *et al*., 1999). The antioxidant, antibacterial and antifungal activities of spices and their derivatives have been investigated by some researchers (De and Banerjee, 1999; Sagdic *et al*., 2003b; Sagdic, 2003). Therefore, the present investigation was carried out to screen of antibacterial potentiality of three spices extracts against pathogenic bacteria in Rossomalai through disc diffusion method for justification of their efficacy as food preservative.

Materials and methods

Collection and storage of spices

Three spices viz. Clove (*Syzygium aromaticum*), Cumin (*Cuminum cyminum*) and Fennel (*Foeniculum vulgare*) were purchased from local market at Rajshahi Metropolitan City, Bangladesh. All samples were dried properly in hot air oven at 50°C for 24h. The dried spices were ground into fine powder using electric blender (IR-091, China) and stored in the dark at room temperature in closed containers until required.

Phytochemical analysis

Phytochemicals of three spices were examined for the presence of saponins, tannins, alkaloids, flavonoids and steroids as described by Parekh and Chanda (2007). The yield of three spices extract was determined as percentage of the quality of following formula: Yield (%) = (Yield/ used spices materials) x 100.

Extraction procedure

Forty gram of spices powder was soaked in 120 ml of ethanol, methanol and sterile water separately to prepare their respective extracts. Then it was shaken vigorously for 72h on orbital shaker (IKA Labortechnik KS 250, Staufen, Germany) to allow for proper extraction and then filtered through sterile thin cloth and Whatman No. 1 filter paper. The resultant juice was evaporated at 80°C in water bath

(HH-S0235, China), after complete evaporation the extracts were preserved aseptically in screw cap tube with respective solvents and prepared 50, 100, 150, 200 and 250 mg ml^{-1} of concentrations superlatively.

Tested bacteria

Three gram negative bacteria i.e. *Escherichia coli* BMLRU1041, *Salmonella* sp. BMLRU1043, *Klebsiella* sp. BMLRU1045 and four gram positive bacteria viz. *Bacillus cereus* BMLRU1028, *Streptococcus* sp. BMLRU1030, *Staphylococcus aureus* BMLRU1032, *Staphylococcus* sp. BMLRU1034 were used as experimental materials which were isolated and identified from Rossomalai sample according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) in Biotechnology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Bangladesh.

Preparation of standard bacterial culture

A loopful colony of 24h surface growth on a nutrient agar plate of each bacterial strain was transferred individually to 5ml of nutrient broth. After incubation at 37°C for 24 h, bacterial cells were collected by centrifugation at 3000 rpm for 15 minutes (Tomos supermini centrifuge,12×1.5ml, 13,400rpm,China) followed by washing twice and resuspended in 0.1% peptone water. Turbidity was adjusted to McFarland turbidity standard approximately 1.5 $\times 10^8$ (CFU ml^{-1}).

Antibacterial assay

The antibacterial activity of three spices extracts was evaluated against seven pathogenic bacteria of rossomalai by disc diffusion assay (Gulluce *et al.*, 2003). Sterile filter paper discs (6 mm) were impregnated in different concentrations (50, 100, 150, 200 and 250 mg ml^{-1}) of each solvent extract separately then air dried aseptically for 5 minutes. Then the discs were carefully placed on seeded plates and incubated at 37°C for 24h. For each extract three replicates were conducted against the test organisms to confirm the reproducible results. Moreover, blank paper discs were impregnated in same amount of solvents as negative control, and ciprofloxacin (0.03 mg ml^{-1}) as positive control. The antimicrobial activity of spices extracts was determined by measuring the zone of inhibition (in mm) against all studied bacteria comparing with control (ciprofloxacin).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC of the three spices extracts were determined according to Doughari *et al.* (2007) for each of the test organisms in triplicate. To 0.5 ml of varying concentrations (5, 25, 50, 75, 100, 125, 150 and 175 $mgml^{-1}$) of the spices extract were added with nutrient broth (2 ml) in test tubes, then a loopful 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 24h. After incubation, the tubes were examined for microbial growth by observing turbidity. The lowest concentration that did not show any growth visually was considered as MIC. The contains of all tubes that showed no visible growth were streaking on nutrient agar plate and incubated at 37°C for 24h. The lowest concentration that did not show any growth of bacterial colony was considered as MBC.

Results

Phytochemical of spices extracts

In qualitative analyses of phytochemical, aqueous extract of three spices showed positive result for saponin, tannin, alkaloid, flavonoid and steroid. A blue-black, brownish-red precipitation, frothing, blue-green ring and pink-tomato red color were indicated the presence of tannin, alkaloid, saponin, terpenoid and flavonoid, respectively (Table 1). The highest yield of extract was measured 6% in *S. aromaticum* aqueous extract and lowest yield was 1.25% in *F. vulgare* methanol extract (Table 1).

Antibacterial assay

The antibacterial activity was varied with the concentration of different spices extracts against different bacteria. Negative control (only solvent) exhibited no zone of inhibition but positive control (30 μg ml^{-1} ciprofloxacin) showed zone of inhibition

against all pathogenic bacteria (Table 2, 3 & 4, Fig. 1 & 2).

In clove inflorescence all the extracts demonstrated antimicrobial activity against tested bacteria. With the ethanol extract demonstrating the highest inhibition zone (15.8 mm) against *E. coli* and lowest (10.8 mm) in *Klebsiella* sp. at 250 mg ml^{-1}. In methanol and aqueous extracts, the highest diameter of inhibition zone was recorded (13.1 mm and 11.8 mm) against *E. coli* and *Salmonella* sp. and the lowest (9.5 mm and 8.6 mm) in *Klebsiella* sp. at same concentration (Table 2, Fig. 1 & 2).

Table 1. Yield and Phytochemicals of spices extract.

Spices	Powder (gm)	Yield of extracts %			Phytochemicals in AQ				
		ET	MET	AQ	S	T	A	F	St
S. aromaticum	40	5.4	3.96	6	+	+	+	+	+
C. cyminum	40	3.3	2.81	3.75	+	+	+	+	+
F. vulgare	40	1.71	1.25	1.81	+	+	+	+	+

ET= Ethanol, MET= Methanol, AQ= Aqueous; + = present, S= Saponins, T= Tannins, A= Alkaloids, F= Flavonoids , St= Steroids.

Table 2. Antibacterial activity of clove inflorescence extracts against pathogenic bacteria in Rossomalai using disc diffusion method.

Extract (mg/ml)	Solvent	Zone of inhibition						
		***Salmonella* sp**	***E. coli***	***Staphylococcus* sp**	***Klebsiella* sp.**	***Bacillus cereus***	***Streptococcus* sp**	***S.* aureus**
50	ET	7.5±0.28	8.4±0.30	0	0	0	0	0
	MET	0	6.8±0.15	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
100	ET	8.9±0.37	10±0.57	7.9±0.26	0	8±0.57	8.1±0.44	0
	MET	7.3±0.44	8.8±0.44	0	0	7.4±0.29	7.5±0.28	0
	AQ	7.2±0.63	7.5±0.28	0	0	0	7.9±0.37	0
150	ET	11.4±0.29	11.5±0.28	10.1±0.66	8.1±0.44	10±0.57	9±0.57	9.5±0.23
	MET	9.6±0.30	9.8±0.44	7.5±0.29	7.1±0.16	8.8±0.60	8.1±0.13	8.1±0.13
	AQ	9.1±0.60	8.8±0.44	7.5±0.36	0	8±0.57	8.1±0.44	7.9±0.21
200	ET	13.5±0.23	13.8±0.60	12±0.57	9±0.57	11.8±0.28	10.4±0.29	11±0.47
	MET	11.5±0.28	12.6±0.88	9.1±0.44	8.5±0.28	10.8±0.44	9.5±0.28	9±0.23
	AQ	10.9±0.26	10.8±0.44	8.5±0.28	7.5±0.28	8.6±0.44	8.8±0.60	8.8±0.36
250	ET	15.1±0.44	15.8±0.44	14.6±0.88	10.8±0.44	14±0.57	12±0.57	13±0.47
	MET	13.1±0.44	13.1±0.60	11.3±0.88	9.5±0.28	11.5±0.28	10.8±0.44	10.5±0.23
	AQ	11.8±0.44	11.8±0.44	9.5±0.28	8.6±0.41	11±0.57	10.5±0.28	10.1±0.36
NC	ET	0	0	0	0	0	0	0
	MET	0	0	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
PC	CF	19±0.57	19.6±0.88	17±0.57	12.8±0.44	15.5±0.28	15±0.57	15.1±0.59

Data are presented as mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic and 0= No zone of inhibition.

Table 3. Antibacterial activity of cumin seed extract against pathogenic bacteria in Rossomalai using disc diffusion method.

Extract (mg/ml)	Solvent	Zone of inhibition						
		Salmonella sp	*E. coli*	*Staphylococcus sp*	*Klebsiella sp.*	*Bacillus cereus*	*Streptococcus sp*	*S. aureus*
50	ET	7.4±0.30	8.5±0.28	0	0	7±0.28	0	0
	MET	0	0	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
100	ET	8.5±0.28	9.8±0.44	0	7.8±0.44	7.6±0.33	8.5±0.28	0
	MET	7.1±0.44	7.5±0.28	0	7±0.28	7±0.28	7.1±0.16	0
	AQ	0	7±0.28	0	6.8±0.16	7.5±0.28	0	0
150	ET	9.5±0.28	11±0.57	9±0.28	9.1±0.16	8.8±0.44	9±0.57	7.1±0.16
	MET	7.6±0.44	8.33±0.33	7.1±0.44	8±0.57	7.8±0.44	8.5±0.28	6.8±0.16
	AQ	7.4±0.34	7.8±0.44	6.6±0.33	7.5±0.28	8.1±0.16	7.5±0.28	0
200	ET	10.8±0.44	13±0.57	10±0.57	11±0.57	11±0.57	11.1±0.60	8.5±0.28
	MET	8.5±0.28	9.5±0.28	8.3±0.33	9.8±0.16	10±0.57	8.6±0.33	8.3±0.33
	AQ	8.4±0.30	8.8±0.44	7.5±0.28	9±0.57	9.5±0.28	8.5±0.28	7.5±0.57
250	ET	13±0.57	15.5±0.28	11.8±0.44	12.5±0.28	13±0.57	12.5±0.28	11±0.57
	MET	10±0.57	11±0.57	10±0.57	10.5±0.28	11.8±0.60	11±0.57	10±0.57
	AQ	10.1±0.44	10.5±0.28	9.8±0.60	10±0.57	11.8±0.44	10.5±0.28	9±0.57
NC	ET	0	0	0	0	0	0	0
	MET	0	0	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
PC	CF	16±0.57	18.1±0.60	14.1±0.72	15±0.57	15.8±0.44	15±0.57	13.8±0.44

Data are presented as mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, 0= No zone of inhibition.

Cumin seed powder extracts showed better result in ethanol extract and displayed the highest inhibition zone (15.5 mm) against *E. coli* and lowest (11 mm) in *S. aureus* at 250 mg ml^{-1}. As for methanol the highest diameter of inhibition zone was recorded (11.8 mm) against *B. cereus* and the lowest (10 mm) was exhibited in both *Salmonella* sp. and *Staphylococcus* sp. On the other hand, in aqueous extract the highest inhibition zone (11.8 mm) was measured against *B. cereus* and lowest (9.0 mm) in *Staphylococcus* sp. at same concentration.

Ethanol extracts of fennel seed showed the highest inhibition zone (15.0 mm) was measured against *E. coli* and lowest (9.5 mm) in *S. aureus* at 250 mg ml^{-1}. In methanol and aqueous extract the highest inhibition zone (14.0 mm and 12.8 mm) was recorded against *B. cereus* and the lowest (9.5 mm and 9.0 mm) was in *S. aureus*.

MIC and MBC of three spices extracts

The MIC values were ranged from 25 to 200 mg ml^{-1}(Table 5). For clove extract, it was ranged from 25 to 75 mg ml^{-1}, for cumin 25 to 100 mg ml^{-1} and 50 to 75 mg ml^{-1} for fennel. Among them clove and cumin ethanol extracts demonstrated lowest value (25 mg

ml^{-1}) against *E. coli*. In case of MBC values, it was ranged from 50 to 225 mg ml^{-1}. Clove and cumin ethanol extracts displayed lowest MBC value (50 mg ml^{-1}) against *E. coli*. But fennel ethanol extract, gave lowest MBC value (75 mg ml^{-1}) against *E. coli* and *B. cereus*.

Table 4: Antibacterial activity of fennel seed extract against pathogenic bacteria in Rossomalai using disc diffusion method.

Extract (mg/ml)	Solvent	Zone of inhibition						
		***Salmonella* sp**	***E. coli***	***Staphylococcus* sp**	***Klebsiella* sp.**	***Bacillus cereus***	***Streptococcus* sp**	***S. aureus***
50	ET	0	7.5±0.28	0	0	7±0.28	0	0
	MET	0	0	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
100	ET	7.1±0.44	8.8±0.44	0	8±0.57	8.5±0.28	8±0.28	0
	MET	7.5±0.28	7.8±0.44	0	7.5±0.28	7.5±0.28	7.3±0.33	0
	AQ	7±0.28	7.1±0.16	0	7.3±0.33	0	0	0
150	ET	8.5±0.28	10±0.57	8.1±0.44	9.5±0.28	10±0.57	9±0.57	8.1±0.16
	MET	7.8±0.16	9.5±0.28	7.8±0.4	8.1±0.16	8.8±0.44	8±0.28	0
	AQ	7.3±0.33	9±0.57	7.5±0.28	8±0.57	7.5±0.28	7.1±0.16	0
200	ET	10±0.57	12.8±0.44	10±0.57	11±0.57	11.5±0.28	11±0.57	10±0.57
	MET	9.5±0.28	11±0.57	9.1±0.16	10±0.57	11±0.57	9.1±0.60	7.5±0.28
	AQ	8.3±0.33	10.5±0.28	8.1±0.16	9.5±0.28	10±0.57	8.3±0.33	7.3±0.33
250	ET	12±0.57	15±0.57	12.8±0.44	13.8±0.44	15±0.57	14.1±0.72	12±0.57
	MET	10.8±0.44	13.8±0.44	11±0.57	12±0.57	14±0.57	11±0.57	9.5±0.28
	AQ	10±0.57	12±0.57	10±0.57	11±0.57	12.8±0.44	10±0.57	9±0.57
NC	ET	0	0	0	0	0	0	0
	MET	0	0	0	0	0	0	0
	AQ	0	0	0	0	0	0	0
PC	CF	15±0.57	18.3±0.88	15.1±0.60	17±0.57	19.8±0.57	17±0.44	14.5±0.28

Data are presented as mean ± SEM of triplicate experiments, AQ= Aqueous, ET=Ethanol, MET= Methanol, NC= Negative control, PC= Positive control, CF= Ciprofloxacin antibiotic, 0= No zone of inhibition.

Table 5. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of three spices extract against pathogenic bacteria.

Spices Extract		Solvent	Bacteria						
			Salmonella sp	*E. coli*	*Staphylococcus sp*	*Klebsiella sp.*	*B. cereus*	*Streptococcus sp*	*S. aureus*
Clove	MIC	ET	50	25	75	125	75	75	125
		MET	75	50	150	150	75	50	125
		AQ	75	100	150	200	125	75	150
	MBC(mg/ml)	ET	75	50	100	150	100	100	150
		MET	100	75	175	175	125	75	150
		AQ	100	125	175	225	175	125	175
Cumin	MIC (mg/ml)	ET	50	25	150	75	50	75	150
		MET	75	75	125	75	100	100	150
		AQ	125	100	125	100	100	125	175
	MBC(mg/ml)	ET	75	50	175	100	100	100	175
		MET	125	125	150	125	125	125	175
		AQ	175	125	150	125	125	150	200
Fennel	MIC (mg/ml	ET	100	50	125	75	50	75	125
		MET	100	75	125	75	75	100	175
		AQ	100	75	150	100	125	125	175
	MBC(mg/ml	ET	125	75	150	100	75	100	150
		MET	125	100	150	125	125	125	200
		AQ	125	125	175	125	175	175	200

ET= Ethanol, MET= Methanol and AQ= Aqueous.

Discussion

The inhibitory effect of three spices extracts on several pathogenic bacteria was investigated. The results revealed that the antibacterial potentiality of these extracts especially the ethanol extract of clove inflorescence was recorded maximum. This is not surprising as the antibacterial nature of spices, in previous work Rahman *et al*. (2011) reported that ginger extract displayed potential inhibitory effect against pathogenic bacteria such as *E. coli*, *Salmonella typhi*, *Listeria* sp., *Bacillus cereus* and *Stephylococcus aureus* in Kachagolla sweetmeat. The relatively high potency of the ethanol extract may be attributed to the dissolving power of alcohols over water (Majorie, 1999).

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). Among the different strains *S. aureus* was least sensitive which suggesting that mechanism of resistance are developing in this organism, where as *E. coli* was more sensitive because extract (ethanol extract of *S. aromaticum*) exhibited highest zone of inhibition against it. In our experiment extracts displayed different growth of inhibition, depending upon bacterial strains, these variations might be due to genetically difference among the strains and this is due to chemical composition and cell wall types of the bacteria (Kaushik and Goyal, 2008). The demonstration of activity both gram-negative and gram-positive bacteria is an indication of broad spectrum activity. In all of the experiments conducted, only solvents used as negative control did not show any appreciable activity, but the standard antibiotic (ciprofloxacin)

consistently displayed superior potency that was different from crude extracts. These differences may be attributed to the fact that ciprofloxacin as a commercial antibiotic is a refined and purified product, while extracts are a mixture of various plant ingredients (El-Mahmood and Doughari, 2008).

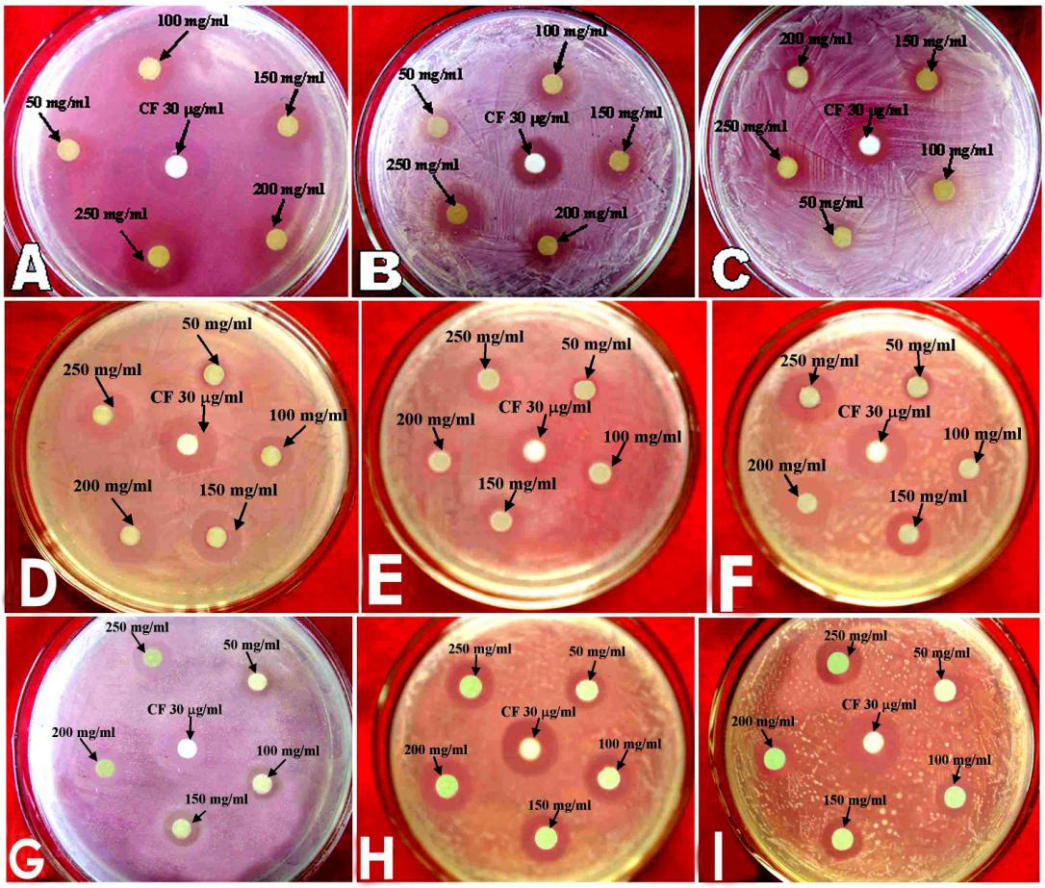

Fig. 1. Antibacterial assay of three spices extract: A, B & C showing zone of inhibition for *S. aromaticum*; D, E & F for *C. cyminum* and G, H & I for *F. vulgare* in ethanol, methanol and aqueous extracts, respectively. [CF = Ciprofloxacin as +ve control.]

Antibacterial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infections and food spoilage. The active component usually interferes with growth and metabolism of microorganisms in a negative manner and is quantified by determining the minimum inhibitory concentration and minimum bactericidal activity (Aboaba *et al*., 2006). Results obtained showed that the MIC value for the most sensitive extract were lower than their MBC value. This suggests that they were bacteristatic at lower concentration but bactericidal at higher concentration. In this study, the low MIC value was exhibited in clove ethanol extract. So it is a good indication of high efficiency of the spice against the bacteria and high MIC and MBC values are indication of low activity (El-Mahmood and Doughari, 2008).

The result showed that three herbal spices viz. *S. aromaticum, C. cyminum, F. vulgare* exhibited more or less inhibitory activity against tested bacteria. Among the spices, *S. aromaticum* showed the best performance. Natural antimicrobial and antioxidant properties of herbal spices play a key role for enhancing shelf-life of foods and controlling food pathogens. So, it can be considered as a promising source of unique natural product to elucidate alternative food preservative.

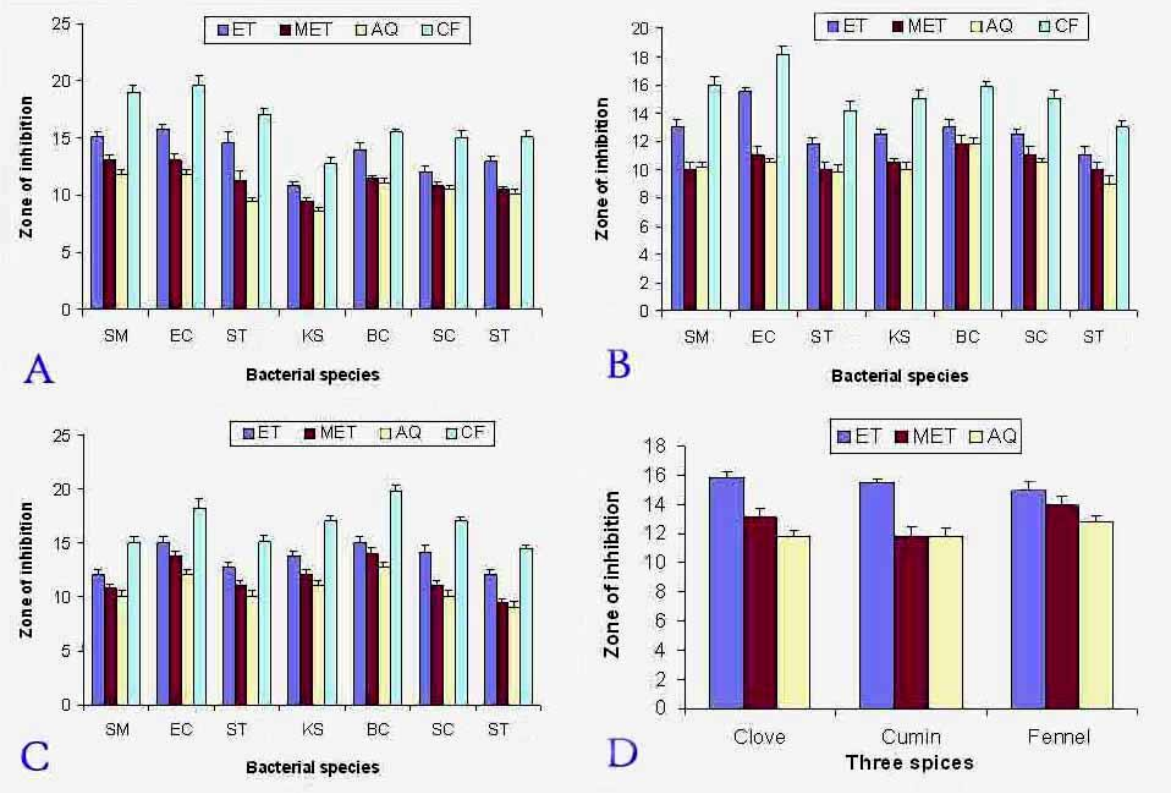

Fig. 2. Showing zone of inhibition against pathogenic bacteria: **A**, **B** & **C** for *S. aromaticum*, *C. cyminum* and *F. vulgare*, respectively. [SM=*Salmonella* sp., EC=*Escherichia coli*, KS=*Klebsiella* sp., BC=*Bacillus cereus*, ST=*Streptococcus* sp., SA=*Staphylococcus aureus*, ST=*Staphylococcus* sp.], and **D.** The highest zone of inhibition of three spices extracts in different solvents at 250 mg ml^{-1}. [ET = Ethanol, MET = Methanol, AQ= Aqueous and CF = Ciprofloxacin.]

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