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RESEARCH ARTICLE

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Antimicrobial activities of combination of different solvent extracts of four Indian spices

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ABSTRACT

In this study, the antimicrobial potential of solvent extract combination (water-acetone, water-ethanol, ethanol-acetone) of four Indian food spices; Mango ginger (Curcuma amada), Black cumin (Nigella sativa), Clove (Caryophyllus aromaticus) and Cinnamon (Cinnamomum verum, synonym C. zeylanicum) were investigated against two microorganisms namely, Bacillus subtilis and Staphylococcus aureus. Each combination of solvent extract was taken in the ration of 1:1, 1:2 and 2:1 separately. Disc diffusion method was used to evaluate antimicrobial activity. The results indicated that the spices have different degrees of bacterial growth inhibition, depending on the strains and combination of solvent extract used.

Key words: Antimicrobial activity, *Curcuma amada*, *Nigella sativa*, *Caryophyllus aromaticus*, *Cinnamomum verum*, Solvent extract, *Bacillus subtilis and Staphylococcus aureus*

INTRODUCTION

In the last few decades, antimicrobial resistance becomes the emerging problem in India as well as all over the world [1,2]. This has gain attention to search for finding new alternative natural antimicrobial agents which will be safer and effective. Moreover, there is a growing demand among consumers for the use of natural preservative or additives in processed foods [3]. Natural additives are mostly preferred in comparison to chemical or synthetic additives because, these are safe, flavor enhancer and devoid of any side effects [4]. Various plant extracts are now becoming popular as natural antimicrobial preservatives or additives [5-7]. Spices are one of the essential additives of most of the Indian cuisine since ancient time. These are used in very small amount to impart flavor, color, taste and aroma in food preparation to improve their palatability [8,9]. Spices are also being used for stabilizing several food items from deterioration [10]. Several studies have been shown that spices are considered to be a richest source of bio-active antimicrobial compounds [11].

The typical Indian spices like Mango ginger (*Curcuma amada*), Black cumin (*Nigella sativa),* Clove (*Caryophyllus aromaticus*) and Cinnamon (*Cinnamomum verum*, synonym *C. zeylanicum*) are commonly used for preparation of curries, pickles, sauces etc. These spices are also being known to have some ethno-medicinal or anti-microbial properties [12]. Antibacterial activities of extract of different plants against various microorganisms have been reported by many scientists [9-15]. Some spices were specifically tested for anti-microbial activities [16-18]. But there are a few report available about antibacterial activities of different solvent extract of some selected Indian spices and herbs [12,18,19,20]. There are hardly any reports available on the synergistic effects of combination of different solvent extract of any spice against common microbes.

In this studies, the antimicrobial activity of combination of different solvent extract as well as different combination rations (water and acetone, water and ethanol, ethanol and acetone) of four common Indian spices such as; Mango ginger (*Curcuma amada*), Black cumin (*Nigella sativa),* Clove (*Caryophyllus aromaticus*) and Cinnamon (*Cinnamomum verum*, synonym *C. zeylanicum*) were investigated against two most common microorganisms, *Bacillus subtilis* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Spice materials: Four typical common Indian spices (Mango ginger, Black cumin, Clove and Cinnamon) in whole stem, seeds, flower and bark respectively were purchased from spice market in Ashokenagar of district north 24 Parganas, West Bengal, India during May, 2013 and sorted for separation of dirt and unwanted materials. The spices were washed thoroughly with sterile double distilled water, sliced and dried in sunlight for five days. The spice samples were then ground to fine powder by a mixture machine. The powder samples were kept in separate containers and preserved for further use.

Chemicals: All the chemicals used in this study were of AR or GR grade and purchased from E. Merck or SRL, India. Nutrient Broth power and Agar power were purchased from Himedia, India.

Preparation of solvent extract of spice powder: 1 g of each dried spice powder was sucked with 5ml of solvent (water or ethanol or acetone) in a closed glass container for 48 hrs at room temperature. The suspension was filtrated with whatman-1 filter paper. The filtrate was collected, dried at 25°C, weighted and re-suspended in double distilled water to a final concentration of 10 mg/ml and stored at 4^oC for further use.

Combination and ratio of solvent extract of spice used in experiment: Ethanol acetone, ethanol water and acetone water solvent extract combination of each spice sample was used in this study. Furthermore, each combination was taken again in three different ratio of 1:1, 1:2 and 2:1 respectively. The final volume of 20µl of combined solvent exact was used for disk diffusion method for assaying antimicrobial activity.

Cultivation and preservation of microbial culture:
The bacterial strains *Bacillus subtilis* (BS) and *Staphylococcus aureus* (SA) were collected from the Department of Biotechnology, Bengal Institute of Technology (BIT), Kolkata, West Bengal. Two strains were cultured in fresh nutrient agar slant [Ingredients in gm/l**:** Peptic digest of animal tissue 5.0**,** Sodium chloride 5.0**,** Beef extract 1.5**,** Yeast extract 1.5, Agar power 2.5%, pH 7.4 ± 0.2] at 37^oC for 24 hrs for preservation in refrigerator (4-6^oC). Active nutrient broth culture (24 hrs grown at 37°C) was used for antimicrobial activity assay.

Spread plate disc diffusion method:
The spread plate disc diffusion method was used to assay antimicrobial activity of spice extract as described by Jorgensen *et al* [21]. In details, 0.2 ml of freshly grown bacterial suspension of each strain was spread on a lawn of nutrient agar containing Petri dish with a sterile bent glass spreader. Sterile whatman no.1 filter paper disc (6-mm diameter) was aseptically placed on plates. 20µl of Spice's combined solvent extract of different ratio (1:1, 1:2 and 2:1) was aseptically poured on each disc along with sterile double distilled water as negative and desired concentration of streptomycin (2.5, 5, 7.5, 10µg/ml) as positive control. Plates were allowed to stand for 30 mins at 4^oC prior to incubation at 37 ºC for 24 hrs. The diameter of zone of inhibition (DIZ) was measured after 24 hrs and represented to nearest mm.

Activity Index determination (A.I):
For the comparative analysis of antimicrobial activity of combined solvent extract of four spices with positive control (Streptomycin) against *Bacillus subtilis* and *Staphylococcus aureus*, the DIZ in mm of positive control having concentration of 10µg/ml was used as reference. The activity index of the antimicrobial activity of the tested combined solvent extract was determined by using the following formula:

The activity index of the combined solvent extract against a specific microbe = [DIZ in mm measured against a sample / DIZ in mm of the reference against same microbes]

Statistical Analysis:
All the analysis was carried out in triplicate and expressed as mean ± SD. The data obtained were subjected to ANOVA test to determine whether there was significant difference between extract used. Differences were considered significant when $p < 0.05$.

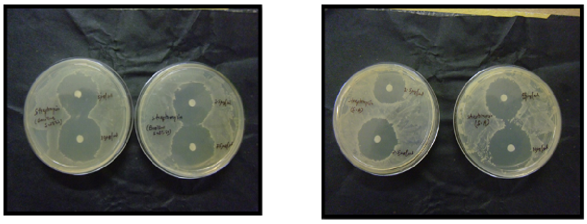
RESULTS AND DISCUSSION

The results of antimicrobial activity of streptomycin of various concentration (2.5µg/ml, 5µg/ml, 7.5µg/ml and 10µg/ml) against *Bacillus subtilis* (*BS*) and *Staphylococcus aureus* (*SA*) are given in Table.1 and Figure.1. The highest DIZ (in mm) against *BS* and SA is found to be 18.3mm and 17.6mm respectively for streptomycin concentration of 10µg/ml.

Table.1: Antimicrobial activity of various concentrations (2.5µg/ml, 5µg/ml, 7.5µg/ml and 10µg/ml) of Streptomycin against *BS* and *SA*

Microorganisms	Concentration of Streptomycin (µg/ml)	DIZ (mm)
Bacillus subtilis	**2.5**	**12**
	5	**15**
	7.5	**17**
	10	**18.3**
Staphylococcus aureus	**2.5**	**13**
	5	**16**
	7.**5**	17
	10	17.6

**DIZ-Diameter of zone of inhibition*



(BS) ***(SA)***

Figure.1: Disc diffusion plates showing DIZ at various concentrations (2.5µg/ml, 5µg/ml, 7.5µg/ml and 10µg/ml) of Streptomycin against BS and SA

The results of antimicrobial activity of different combination of solvent extract of Mango ginger (*Curcuma amada*) are represented in Table.2 and Figure.2. From these results, it is observed that water acetone extract combination did not show any measurable DIZ for all combination ratios (1:1, 1:2 and 2:1) against both BS and SA in comparison to water ethanol and ethanol acetone extract combination. All ethanol acetone extract combination ratios have shown relatively greater activity index (AI) in comparison to water ethanol. The highest activity index (AI) against BS is measured to be 0.490 for 1:2 ratio of combination of ethanol acetone extract of Mango ginger, whereas the same against SA is 0.379 for 1:2 ratio of combination of ethanol acetone extract.

Table.2: Antimicrobial activity of combination of different solvent extract (water, ethanol and acetone) of Mango Ginger against *BS* and *SA*

Combined solvent extract used for microbial assay	Ratio of extract used for microbial assay	DIZ (mm)			
		S.A		B.S	
		DIZ	A.I	DIZ	A.I
Water + Acetone	1:1	N.I	-	N.I	-
	1:2	N.I	-	N.I	-
	2:1	N.I	-	N.I	-
Water+ Ethanol	1:1	N.I	-	3.3	0.202
	1:2	3.6	0.216	4.6	0.282
	2:1	4.3	0.259	4	0.245
Ethanol+ Acetone	1:1	4.3	0.259	5	0.306
	1:2	6.3	0.379	8	0.490
	2:1	3.6	0.216	4.3	0.263

**DIZ-Diameter of zone of inhibition, *S.A-Staphylococcus aureus, *B.S-Bacillus subtilies, *N.I-No inhibition zone, *A.I- Activity index*

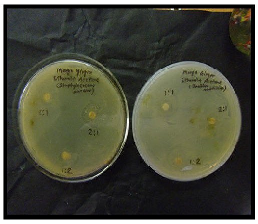


Figure.2: Disc diffusion plate showing DIZ of ethanol acetone extract combination of Mango ginger against BS and SA

All water acetone and ethanol acetone extract combinations of Black cumin (Nigella sativa) have shown considerably better antimicrobial activity (Table.3 and Figure.3) against both SA and BS in comparison to water ethanol combination. From the results, it is observed that all water acetone extract combinations (1:1, 1:2 and 2:1) have shown comparatively highest potential of antimicrobial activity in terms of AI against both BS and SA. The highest AI against BS is measured to be 0.429 for 2:1 ratio of water acetone extract combination of Black cumin whereas the same against SA is measured to be 0.783 for 2:1 ratio of water acetone extract.

Table.3: Antimicrobial activity of combination of different solvent extract (water, ethanol and acetone) of Black cumin against *BS* and *SA*

Combined solvent extract used for microbial assay	Ratio of extract used for microbial assay	DIZ (mm)			
		S.A		B.S	
		DIZ	A.I	DIZ	A.I
Water + Acetone	1:1	8.3	0.5	6.3	0.386
	1:2	6.3	0.379	6.6	0.404
	2:1	13	0.783	7	0.429
Water+ Ethanol	1:1	N.I	-	4.6	0.282
	1:2	N.I	-	4.3	0.263
	2:1	N.I	-	N.I	-
Ethanol+ Acetone	1:1	4.3	0.259	5	0.306
	1:2	3.6	0.216	5	0.306
	2:1	3.3	0.198	5.3	0.325

**DIZ-Diameter of zone of inhibition, *S.A-Staphylococcus aureus, *B.S-Bacillus subtilies, *N.I-No inhibition zone, *A.I- Activity index*

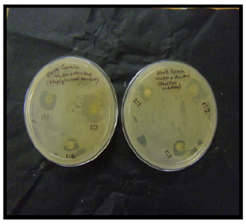


Figure.3: Disc diffusion plate showing DIZ of water acetone extract combination of Black cumin against BS and SA

Table.4: Antimicrobial activity of combination of different solvent extract (water, ethanol and acetone) of Clove extracts against *BS* and *SA*

Combined solvent extract used for microbial assay	Ratio of extract used for microbial assay	DIZ (mm)			
		S.A		B.S	
		DIZ	A.I	DIZ	A.I
Water + Acetone	1:1	N.I	-	N.I	-
	1:2	5.3	0.319	N.I	-
	2:1	4.3	0.259	N.I	-
Water+ Ethanol	1:1	4.6	0.277	5.3	0.325
	1:2	4	0.240	4.6	0.282
	2:1	5.3	0.319	N.I	-
Ethanol+ Acetone	1:1	7	0.421	4.3	0.263
	1:2	4.6	0.277	4.3	0.263
	2:1	4.6	0.277	6	0.368

**DIZ-Diameter of zone of inhibition, *S.A-Staphylococcus aureus, *B.S-Bacillus subtilies, *N.I-No inhibition zone, *A.I- Activity index*

Clove (*Caryophyllus aromaticus*) is another most commonly used spices in India. All solvent extract combinations of Clove (*Caryophyllus aromaticus*) have shown considerable antimicrobial activity (Table.4 and Figure.4). All ethanol acetone extract combinations (1:1, 1:2 and 2:1) considered to have higher antimicrobial potential in comparison to other solvent extract combination against both BS and SA (Table.4 and Figure.4). The highest activity index for 2:1 ratio of ethanol acetone extract of Clove is measured to be 0.368 against BS whereas the same against SA is measured 0.421 for the 1:1 ratio of ethanol acetone extract.

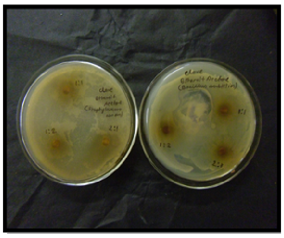


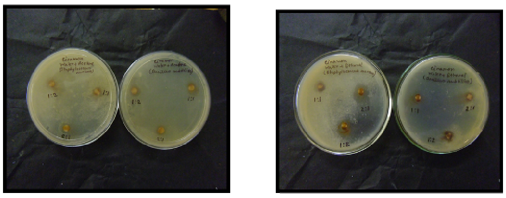
Figure.4: Disc diffusion plate showing DIZ of ethanol acetone extract combination of Clove against BS and SA

The antimicrobial activities of solvent extract of Cinnamon (Cinnamomum verum, synonym C. zeylanicum) are represented in Table.5 and Figure5. It is clear from these results that all ratios of ethanol acetone extract combination (1:1, 1:2 and 2:1) generates significant DIZ against both BS and SA (Table.5 and Figure.5) in comparison to water acetone and water ethanol extract combination. The 1:1 ratio of water acetone extract of Cinnamon have shown highest activity index (0.386) against BS whereas the same against SA is measured to be 0.481 by the 2:1 ratio of water ethanol extract combination.

Table.5: Antimicrobial activity of combination of different solvent extract (water, ethanol and acetone) of Cinnamon extract against *BS* and *SA*

Combined solvent extract used for microbial assay	**Ratio of extract used for microbial assay**	**DIZ (mm)**			
		S.A		B.S	
		DIZ	A.I	DIZ	A.I
Water + Acetone	1:1	5.3	0.319	6.3	0.386
	1:2	N.I	-	5	0.306
	2:1	N.I	-	4	0.245
Water+ Ethanol	1:1	4.6	0.277	N.I	-
	1:2	6.6	0.397	N.I	-
	2:1	8	0.481	5	0.306
Ethanol+ Acetone	1:1	4.3	0.259	4.3	0.263
	1:2	6.3	0.379	4.6	0.282
	2:1	6.3	0.379	3	0.184

**DIZ-Diameter of zone of inhibition, *S.A-Staphylococcus aureus, *B.S-Bacillus subtilies, *N.I-No inhibition zone, *A.I- Activity index*



(WA) (WE)

Figure.5: Disc diffusion plates showing DIZ of water acetone (WA) and water ethanol (WE) extract combination of Cinnamon against BS and SA

Growing concern about food safety it has recently led to the development of natural antimicrobials to control food spoilage microorganisms [22]. In the present study, combined solvent extract was preferred to check the antimicrobial activity of the four common Indian spices which are used frequently for the preparation Indian dishes. Combination of solvent extract of spices in several cases [23] demonstrated synergistic or additive effects on microorganisms. According to Cain [24], synergistic activity suggested different mode of action of the combining compounds. It was reported that Mango ginger contains a new antimicrobial compound known as difurocumenonol [25], which has highest activity against gram-positive bacteria. According to Cowan [26], high resistance of gram-negative bacteria to Mango ginger extracts may be due to the presence of outer layer which is composed of lipopolysaccharides, and which acts as an effective barrier for many hydrophobic molecules. In the present study it was observed that combined solvent extract of Mango ginger did not generate any antimicrobial properties (Table.2) against two popular gram positive microorganisms, *S. aureus* and *B. subtilis* which is rather contradictory according to the previous results [25,26]. Chloroform and ethanol extract of Black cumin seed (*Nigella sativa*) was analyzed for antibacterial activity against *S. aureus* and *B. cereus,* which was previously found to be resistant to different antibiotics [27]. In the present study, water acetone and ethanol acetone extract combination of Black cumin seed

(*Nigella sativa*) have showed greater antimicrobial property (Table.3) against the strains *S. aureus* and *B. subtilis.* It was reported that methanolic extract of Clove was more effective antimicrobial property against gram positive culture, *S. aureus* in comparison to ethanolic extract [18]. Our study demonstrated that water acetone and water ethanol extract combination of Clove exerted better antimicrobial activity (in terms of AI, Table.4) against both *S. aureus* and *B. subtilis* strains. The antimicrobial activity of cinnamon is due to the presence of cinnamaldehyde, an aromatic aldehyde that inhibits amino acid decarboxylase activity and has been proven to be active against many pathogenic bacteria [28]. The electronegative behavior of cinnamaldehydemay interfere in biological processes involving electron transfer and react with nitrogen-containing components, e.g. proteins and nucleic acids, and therefore inhibit the growth of the microorganisms [29]. Researchers evaluated antibacterial activities of ethanol and acetone extract of Cinnamon bark against *S. aureus* and *B. subtilis*. It was reported that acetone extract of cinnamon did not shown any antimicrobial activity against *S. aureus* and *B. subtilis* [30]. But in the present study it was observed that water acetone, water ethanol and ethanol acetone extract combination of Cinnamon bark exhibited considerable antimicrobial property (in terms of AI in Table.5) against *S. aureus* and *B. subtilis*. These finding may be due to synergistic effect of combination of solvent extract of Cinnamon which may not generate sole solvent extract.

Among the various compounds present in solvent extract of spices, phenolic, terpenoids, and alkaloids are very important compound that exhibited antimicrobial or antioxidant effects [31,32]. Further studies are required to determine the effective antibacterial compounds from these spices and their full spectrum of efficacy. These ethno-medicinal spices or their combination will open the prospect of finding new clinically efficient antimicrobial compound.

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