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Synergistic or additive antimicrobial activities of Indian spice and herbal extracts against pathogenic, probiotic and food-spoiler micro-organisms

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Abstract: Traditionally the people of India have a long-standing practice of using wide variety of herbal products in treatment of diseases or as preservatives in foods. Spices are indispensable components of Indian cuisines since ancient times. Spices are considered as rich source of bio-active antimicrobial compounds. The disc diffusion and MIC bioassays were performed with some selected Indian spices and herbs against some entero-pathogenic, probiotic or food-spoiler microbes. Widest inhibition zones (12-14 mm DIZ) were seen in cases of aqueous extracts of fenugreek, mustard and henna. Gram positive bacteria were more prone to these spices or herbal extracts than Gram negative bacteria and fungus. *Klebsiella pneumonie* and *Aspergillus niger* were the most resistant microbes while *Staphylococcus aureus* and *E. coli* were most susceptible strains. Combinations of the spices in several cases demonstrated synergistic or additive effect. No antagonistic effect was seen. Cumin and fenugreek or Black cumin and mustard combinations demonstrated higher synergistic antimicrobial effects.

Keywords: Antimicrobial, spice, synergy, additive, MIC, FIC

Introduction

In the last two decades, antibiotic resistance is an emerging problem worldwide (Walsh, 2000; Cohen, 2002). This has lead to the search for new, safe and effective antimicrobial agents from alternative natural resources like plant products. At the same time, there is a growing demand among consumers for natural preservative or additives in processed foods (Gutierrez *et al*., 2008). In comparison to chemical or synthetic additives herbal additives are preferred as these are safer, flavour enhancer and without any side effects (Brull and Coote, 1999). Herbal extracts are fast becoming popular as natural antimicrobial preservatives or additives (Akarpat *et al*., 2008; Pazos *et al*., 2008; Cox *et al.*, 2010).

Traditionally the people of India have a long-standing practice of using extensive diversity of plant products in treatment of diseases. Spices are essential components of Indian cuisines since ancient times. These are used in minute amounts to impart flavour, taste and aroma in food preparation to improve their palatability (Rahman and Gul, 2002; Nair and Chanda, 2006). Spices are also used for stabilizing several food items from deterioration (Kizil and Sogut, 2003). Spices are considered as rich source of bio-active antimicrobial compounds (Lia and Roy, 2004). The typical Indian spices and herbs like cumin, black cumin, mustard, fenugreek, ajowain, curry-leaf, nutmeg and henna are usually used in curries, pickles, sauces etc. These spices are also known to have some ethno-medicinal or anti-microbial properties (Singh *et al*., 2002). Plants traditionally used for medicinal purpose in different parts of the world have been screened for possible antimicrobial action by several workers (Bonjar, 2004). Antibacterial activities of extracts of different plants against various microorganisms have been reported by many scientists (Sagdic and Ozcan, 2003; Nair and Chanda, 2006; Shan *et al*., 2007; Chaudhury and Tariq, 2008; Gutierrez *et al*., 2008). Some medicinal herbs have also been assessed (Ahmad and Beg, 2001). Some spices were specifically tested for anti-microbial activities (Shelef, 1983; Sagdic *et al*., 2003). But there are little reports on some of the Indian spices and herbs (Singh *et al*., 2002; Arora and Kaur, 1999; Romson *et al*., 2011). There is no report on their synergistic effects especially on food-spoiler and probiotic microbes. The objective of the present study is to evaluate some traditional Indian spices and their combining effects on selected pathogenic, food-spoiler and probiotic microorganisms.

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Materials and Methods

Plant materials

Eight types of typical Indian spices and herbs (cumin, black cumin, mustard, fenugreek, ajowain, curry-leaf, nutmeg and henna) (in whole fruit, seed or leaves form) were purchased from reliable retail shop in Kolkata in April-June, 2009. The spices were dry fruits of *Cuminum cyminum* L. (Apiaceae) (Cumin) and *Trachyspermum ammi* L. (Apiaceae) (Ajowain); seeds of *Nigella sativa* L. (Ranunculaceae)(Black cumin), *Brassica nigra* L. (Fabaceae)(Mustard) and *Trigonella foenumgraceum* L. (Fabaceae) (Fenugreek); dry leaves of *Lawsonia inermis* L., (Lythraceae)(Henna) and *Murraya koenigii* L. (Rutaceae) (Curry-leaf) and nuts of *Myristica fragrance* Houtt. (Myristicaceae) (Nutmeg). The dry spices were sieved and checked visually for any contamination.

Preparation of aqueous decoction

The dried spices were washed thoroughly with sterile double distilled water to make these spices completely free from any possible contamination. Aqueous decoction of each spice was prepared by boiling 20 g of dry spice in 100ml sterile distilled water over moderate flame for 20 min. The aqueous extract was cooled, filtered through Whatman No.1 filter paper and then kept in sterile screw capped glass vials at 4ºC. The aqueous extracts were re-confirmed as free of any contamination by plating method (APHA, 1992). These crude aqueous decoctions were diluted with sterile double distilled water (which is to be used as negative control) to obtain required concentrations before experiments.

Preparation of solvent extraction

Twenty gram of each dry sample was crushed in ethanol for 48 hours at 24ºC with stirring (Liu and Nakano, 1996). The extracts were centrifuged and filtered through Whatman No.1 filter paper and evaporated using vacuum rotary evaporator to near dryness and stored in glass vials in dark at 4ºC. These crude solvent extracts were diluted with 10% dimethyl sulphoxide (DMSO- which is to be used as negative control) to obtain required concentration before experiments.

Test organisms

Four enteropathogenic, three food-spoiler and one probiotic bacterial strains were selected for the study. The enteropathogenic and food-spoiler strains were taken to assess antimicrobial activities of spices against those strains. To understand the interference of spices against the growth of beneficial gut-bacteria, the probiotic strain was included in this study. The strains were *Salmonella enterica* serovar typhimurium MTCC 3224, *Serratia marcescens* MTCC 4822, *Staphylococcus aureus* MTCC 7405, *Escherichia coli* MTCC 3221, *Klebsiella pneumoniae* subsp pneumoniae MTCC 6644, *Proteus vulgaris* MTCC 7299, *Bacillus cereus* MTCC 6909, *Lactobacillus brevis* MTCC 4460 were obtained from MTCC, IMTECH, Chandigarh, India. All bacterial cultures were maintained on tryptic soy agar (HiMedia) and subcultured regularly. The fungal strain *Aspergillus niger* was taken from laboratory collection (isolated from bread) and grown on Sabouraud dextrose agar (HiMedia). Standard inoculum was prepared by sub-culturing 4-5 freshly grown isolated colonies of each strain in Tryptic soy broth (TSB) and incubated at 35-37 ºC for 24 hours. Inocula were standardized with sterile TSB to give final cell load of 10^6-10^7 CFU/ml.

Disc diffusion bioassay

The disc diffusion test was performed as described by Jorgensen *et al.* (1999). A 0.5 ml standardized inoculum suspension of each bacterial strain was spread on TSA plates with a sterile bent glass rod spreader. Sterile 6-mm Whatman no.1 filter paper discs were aseptically placed on plates. Spice decoctions or extracts of standard concentrations (10 mg dry weight) were aseptically poured on the discs along with sterile double distilled water or 10% DMSO as negative and ampicillin as positive controls. Plates were allowed to stand for 30 minutes at room temperature prior to incubation at 35-37 ºC for 24 hours. The inhibition zone diameters were measured three times and means were represented to nearest mm.

Determination of MICs

Minimum inhibitory concentrations (MICs) were determined by broth dilution method in culture tubes (Jorgensen *et al.*, 1999). Various concentrations (50, 40, 30, 25, 20, 15, 10, 7.5, 5, 2.5, 1.25 mg dry weight/ml) of the extracts were added to broth immediately after inoculating with fresh 0.2 ml culture of the strain, keeping final volume at 5 ml. The cultures were incubated on a rotary shaking incubator at 37ºC for 48 hours. The lowest concentration of the spice or herbal extracts showing no visible growth was considered as the MIC.

Combined effect study

The more effective spices were chosen for combined assessment against selected micro-organisms. The spices were combined on one to one basis as popularly used in conventional Indian cooking or fresh fast food preparations. The assessment was done using checkerboard assay method (Satish *et al*., 2005). The combined effect of spices was calculated by the following formula and results were interpreted as synergy (S, FIC≤ 0.5), addition (A, 0.5<FIC<1), indifference (I, 1<FIC<4) and antagonism (AN, FIC≥4) (Berenbaum, 1981; Gutierrez et al., 2009):

$\sum FIC = FIC_{A(comb)} + FIC_{B(comb)}$

=MIC of A in combination/ MIC_A+ MIC of B in combination/MIC_B

Statistical analyses

The experiments were done at least twice and their mean values were represented. All statistical analyses including ANOVA were done in SPSS Version 17.0. Differences were considered significant when $p< 0.05$.

Results and Discussion

In India, spices are ethnically used as active ingredients in ayurvedic medicines and reported to possess a number of pharmacological effects to treat different human ailments (Bonjar *et al*., 2004). Several investigations have been directed towards their anti-microbial properties (Voravuthikunchai *et al*., 2005). The disc diffusion assay showed that the spices have different degrees of bacterial and fungal growth inhibition, depending on the strains (Table 1). The aqueous extracts of spices like cumin, mustard and ajowain showed broadest antimicrobial activity by inhibiting more or less most of the microbial strains involved. Ethanolic extracts of cumin, fenugreek and curry-leaf indicated higher anti-microbial activity showing greater diameter of inhibition zones. In case of black cumin or henna, aqueous extract was more effective than their ethanolic extracts. But in cases of curry leaf, ethanolic extract was stronger. Widest inhibition zones (12-14 mm DIZ) were seen in cases of aqueous extracts of fenugreek, mustard and henna. Gram positive bacteria were more prone to these spices or herbal extracts than Gram negative bacteria and fungus. *Staphylococcus aureus* was found to be the most sensitive strain. *Klebsiella pneumonie* was the most resistant strain tested against these spices. It is clear that different extracts or decoctions of spices or herbs differ in their anti-microbial activities, which may depend on solubility of the active constituents.

Table 1. Antibacterial activities, indicated by diameter of inhibition zone (DIZ, mm, for 10 mg dry wt./ disc, Mean±SD) of selected spices or herbs against the micro-organisms [- means <6mm DIZ]

Spices	Extract	*Escheri-chia coli*	*Staphylo-coccus aureus*	*Salmonella enterica*	*Serratia marcescens*	*Klebsiella pneumo-niae.*	*Proteus vulgaris.*	*Bacillus cereus*	*Lacto-bacillus brevis*	*Aspergillus niger*
Cumin	*Aqueous*	10±2	11 ±2	-	7±1	9±1	11±3	10±2	8±1	8±2
	Ethanol	9±1	11±2	8±2	10±3	7±1	9±1	9±3	-	-
Blackcumin	*Aqueous*	8±2	10±1	-	-	8±1	9±1	-	6±1	-
	Ethanol	-	9±3	8±3	-	-	-	6±2	8±1	-
Mustard	*Aqueous*	12±3	9±2	9±2	11±3	-	12±2	12±3	11±2	12±3
	Ethanol	6±2	7±2	9±2	7±2	-	-	8±2	6±2	11±2
Fenugreek	*Aqueous*	12±2	14±3	-	-	-	9±2	10±3	7±2	10±2
	Ethanol	9±2	11±2	7±2	8±3	-	7±2	9±2	-	6±2
Ajowain	*Aqueous*	10±2	12±2	9±2	10±2	7±2	7±2	11±3	9±2	8±3
	Ethanol	-	8±2	9±2	-	-	-	6±2	-	-
Henna	*Aqueous*	11±2	13±3	6±1	-	-	6±2	13±2	10±2	6±2
	Ethanol	-	-	-	-	-	-	6±1	6±2	-
Nutmeg	*Aqueous*	8±2	11±3	-	6±1	6±2	7±2	9±3	7±1	7±3
	Ethanol	10±2	6±2	-	-	6±1	8±3	-	6±1	-

Curry leaf	*Aqueous*	6±1	-	8±2	8±2	-	-	-	-	-
	Ethanol	8±3	7±2	10±2	7±2	-	8±2	7±2	-	7±3
Ampicillin		15±2	16±3	11±2	15±2	9±2	12±2	11±3	10±2	-

Table 2. Antibacterial activities, indicated by minimum inhibitory concentrations (MIC, mg dry wt. ml^{-1}) of selected spices or herbs against the micro-organisms

Spices	*Extract*	*Escheri-chia coli*	*Staphylo-coccus aureus*	*Salmonella enterica*	*Serratia marcescens*	*Klebsiella pneumo-niae.*	*Proteus vulgaris*	*Bacillus cereus*	*Lacto-bacillus brevis*	*Aspergillus niger*
Cumin	*Aqueous*	10	15	>50	15	10	10	10	20	30
	Ethanol	15	20	25	20	25	20	40	40	50
Blackcumin	*Aqueous*	25	15	40	40	20	15	50	50	>50
	Ethanol	>50	25	40	50	>50	50	40	30	40
Mustard	*Aqueous*	10	20	15	15	40	10	15	30	15
	Ethanol	40	40	30	30	>50	50	30	15	15
Fenugreek	*Aqueous*	15	5	40	40	50	20	20	40	15
	Ethanol	25	20	40	15	50	30	40	50	40
Ajowain	*Aqueous*	10	7.5	20	25	15	25	15	30	25
	Ethanol	40	30	20	40	50	50	40	50	40
Henna	*Aqueous*	10	5	30	50	50	40	10	15	50
	Ethanol	30	40	50	50	>50	50	30	40	50
Nutmeg	*Aqueous*	25	15	50	40	25	20	20	40	30
	Ethanol	20	25	50	>50	40	20	40	40	>50
Curry leaf	*Aqueous*	50	40	30	25	50	50	40	40	50
	Ethanol	30	30	25	50	50	40	40	50	40
Ampicillin		7.5	5	5	5	7.5	10	10	7.5	50

Table 3. Antibacterial activities, indicated by Fractional inhibitory concentrations ($FIC_A+FIC_B=\sum FIC$, mg dry wt. ml^{-1}) of combined spices or herbs against selected micro-organisms (S, FIC≤ 0.5), addition (A, 0.5<FIC<1), indifference (I, 1<FIC<4) and antagonism (AN, FIC≥4)

Combined Spices (A+B)	Extract	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella enterica*	*Serratia marcescens*	*Klebsiella pneumoniae.*	*Proteus vulgaris*	*Bacillus cereus*	*Lactobacillus brevis*	*Aspergillus niger*
Cumin + Fenugreek	*Aqueous*	0.75+0.6= 1.42 (I)	0.83+0.5= 0.83 (A)	-	-	-	0.25+0.25= 0.5 (S)	0.5+0.25= 0.75 (A)	-	0.5+ 0.33= 0.83 (A)
	Ethanol	1.0+1.0= 2.0 (I)	0.75+ 0.5= 1.25 (I)	-	0.5+0.33= 0.83 (I)	-	-	-	-	-
Black cumin + Mustard	*Aqueous*	0.8+1.0= 1.8 (I)	0.17+0.25= 0.42 (S)	-	-	-	0.33+0.5= 0.83 (A)	-	-	-
	Ethanol	-	-	0.5+0.5= 1.0 (A)	-	-	-	-	0.67+0.33= 1.0 (A)	0.75+0.67= 1.42 (I)
Mustard + Curry leaf	*Aqueous*	-	-	0.67+0.6= 1.34 (I)	0.67+0.8= 1.47 (I)	-	-	-	-	-
	Ethanol	-	-	0.5+0.4= 0.9 (A)	-	-	-	-	-	1.0+0.67= 1.67 (I)
Cumin + Ajowain	*Aqueous*	0.5+0.5= 1.0 (A)	0.67+1.0= 1.67 (I)	-	-	1.0+1.33= 2.33 (I)	-	0.75+0.6= 1.42 (I)	-	-
	Ethanol	-	-	0.75+0.75= 1.5 (I)	-	-	-	-	-	-

The MIC assay of aqueous and ethanolic extracts showed that cumin had the highest anti-microbial action against the strains tested, followed by mustard, henna and ajowain (Table 2). Whether the aqueous or ethanolic extract would work well on microbes, it all depends on active constituents (de Boer *et al*., 2005). Among the microbial strains tested, *Klebsiella pneumonie* and *Aspergillus niger* were the most resistant microbes while *Staphylococcus aureus* and *E. coli* were the most susceptible strains. Spices like cumin, fenugreek or henna worked very well against *E. coli* though *E. coli* was earlier shown as resistant to different anti-microbial agents (Saeed *et al*., 2007). Probiotic strain *Lactobacillus brevis* was to some extent resistant to spices so it may be expected that these spices would not interfare with growth of *Lactobacillus brevis* in human gut. Micro-organisms differ in their resistance to a given spice.

Oneway Analysis of Variance was used to determine whether the antimicrobial activity differs among different type of spices. The analysis shows significant difference among the groups $F(8,72)=4.254$, $p<0.01$). Post Hoc Tamhane test shows that Curry leaf differ significantly from other groups and is statistically significant for Cumin, Ajowin and ampicillin. ANOVA, in case of ethanolic extracts shows significant difference among the groups $F(8,72)=6.263$, $p<0.01$). Post Hoc Scheffe test shows that Ampicilin differ significantly from other groups and is statistically significant for blackumin, ajowain, henna, nutmeg curry leaf.

Combined antimicrobials are preferred as microbial tolerance is less likely to develop against substances having more than one type of modes of action (Gutierrez *et al*., 2008). It was thus necessary to check the antimicrobial activities of these spices in combinations as used in conventional cooking or salad dressing. Combinations of the spices in several cases demonstrated synergistic or additive effects on microorganisms and showed lower FICs (Table 3). Combinations like aqueous extract of cumin and fenugreek showed synergistic activity against *Proteus vulgaris* and additive effects against *Staphylococcus aureus, Bacillus cereus* and *Aspergillus niger.* Black cumin and mustard demonstrated synergistic activity against *Staphylococcus aureus* and additive antimicrobial effects against *Salmonella enterica, Proteus vulgaris and Lactobacillus brevis*. Synergistic or additive effects support the use of these spices in combination in stead of use in isolation. According to Cain *et al*. (2003) synergistic activity suggests different mode of actions of the combining compounds. Combinations like mustard and curry leaf or cumin and ajowain generally showed indifferent effects. No combinations showed antagonistic effect. Toroglu (2011) showed how some spice essencial oils showed synergistic activities with antibiotics. The effective spice-combinations may be engaged in food preservation and may lead to new choices for antimicrobial agents. It could be concluded that cumin, mustard and henna have potentially higher antimicrobial efficacy. All tested herbal extracts (aqueous or ethanolic) have more or less antimicrobial efficacy against all microbes examined. When used in combinations these spices generally show synergistic antimicrobial effect specially on fungus.

Differential antimicrobial activity of herbs against different bacteria might be due to present of different active phyto-compounds. Among those antimicrobial compounds, phenolic compounds, terpenoids, and alkaloids are very important compounds in antimicrobial or antioxidant effects (Hoult and Paya, 1996; Rios and Recio, 2005). Some of the known active constituents are cuminaldehyde and monoterpene hydrocarbons like β-pinene of cumin, thymol of black cumin, sinigrin glucoside of mustard, trigonelline alkaloid of fenugreek, volatile terpenes and thymol of ajowain, monoterpene hydrocarbons like α-pinene of nutmeg and polyphenols of curry-leaf and henna (Karapinar and Aktug, 1987; de Guzman and Siemonsma, 1999). Further study is required to determine the different antibacterial compounds from these herbs and their full spectrum of efficacy. These ethno-medical spices and herbal resources or their combinations open the prospect of finding new clinically efficient antimicrobial compounds. The knowledge about the botanical preparation of traditional medicinal plants can be extended for future investigation into the field of pharmacology, phyto-chemistry or food chemistry for better drug discovery.

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