

Antimicrobial activity of individual and combined extracts of selected spices against some pathogenic and food spoilage microorganisms

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Abstract

Spices are indispensable components of Indian cuisines since ancient times and are considered as rich source of bio-active antimicrobial compounds. Antibacterial and antifungal activity of individual as well as ethanolic extracts of cumin (*Cuminum cyminum*), ginger (*Zingiber officinale*) and garlic (*Allium sativum*) was evaluated against bacterial strains of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Salmonella* Typhi and fungal strains of *Candida albicans* and *Rhizopus azygosporus*. Agar well diffusion assay for antimicrobial activity yielded the inhibitory zone of 12.8 to 18.3 mm diameter for cumin, 11.5 to 16.3 mm diameter for ginger and 16.8 to 19.3 mm diameter for garlic extract indicating that garlic was the most effective spice in inhibiting the microbial growth. The combined extracts showed inhibition zones ranging from 12.3 to 19.6 mm in diameter against bacteria and 15.6 to 19.6 mm against fungus. The combined extract of cumin and garlic was found to be most effective in inhibiting the microbial growth. The MIC of individual extracts was 12.5 mg/ml against all the tested microorganisms. The MIC of combined extracts fluctuated from 3.8 to 6.7 mg/ml and the most sensitive microbial species in relation to the MIC of combined extracts was *S. Typhi*. The fractional inhibitory concentrations (FIC) values of the combined extracts suggested additive inhibitory effect of the combined spice extracts ($0.5 \leq \text{FIC index} \leq 1$).

Keywords

Antimicrobial

Spice extracts

Additive

MIC

FIC

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Introduction

Uncontrolled use of chemical antimicrobial preservatives has been the inducing factor for appearance of more and more microbial strains resistant to classic antimicrobial agents (Kießling *et al.*, 2002). Increasing use of chemicals antimicrobials have created a situation leading to an ecological imbalance and enrichment of multiple multi-resistant pathogenic microorganisms. Consumers demand has also increased for foods having long shelf-life but with presence of minimum or no chemical food additives. Therefore, there has also been a growing demand among consumers for natural preservatives or additives in processed food (Gutierrez *et al.*, 2008). This perspective has put pressure on the food industry for progressive removal of chemical preservatives and adoption of natural alternatives to obtain its goals concerning microbial safety. As compared to chemical or synthetic additives herbal additives are preferred as these are safer, flavor 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; Cox *et al.*, 2010). Therefore, it has given an impetus to the research activities related to the novel antimicrobial

compounds from natural sources especially culinary spices and herbs. Plants, including herbs and spices, contain products of secondary metabolism such as phenolics, phenolic acids, quinones, flavonoids, tannins (Burt, 2004).

The typical Indian spices and herbs like cumin, garlic, ginger, mustard, fenugreek, ajowain, curry-leaf, nutmeg etc. are usually used in curries, pickles, sauces etc. Mostly spices are known to have some ethno-medicinal or anti-microbial properties (Singh *et al.*, 2002). Active compounds of spices have been included in class of naturally occurring food preservatives (Brull and Coote, 1999). Inhibitory effect of spices on a variety of microorganisms has been reported, although considerable variation for resistance of different microorganisms to a given spice and of the same microorganisms to different spices has also been observed (Akgul and Kivanç, 1988). *Allium sativum*, known to most as garlic is known for having an array of antiviral, anti-fungal, and antibacterial properties. Along with its protective abilities, allicin is believed to be the natural chemical component responsible for the antimicrobial effects of garlic. Various studies have shown that garlic is known to be effective against gram-negative as well as gram-positive bacteria, such as *Escherichia coli*,

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Salmonella, *Staphylococcus*, and *Streptococcus* species (Waqar et al., 1994; Kumar and Berwal, 1998; Arora and Kaur, 1999; Sivam, 2001; Cutler and Wilson, 2004; Sarma, 2004; Joe et al., 2009; Iram et al., 2012; Ismail et al., 2012; Virendra et al., 2013). Ginger has also been shown to be effective against the growth of both gram-positive and gram-negative bacteria. The main active phytochemicals present in ginger are gingerols, shogaols and paradols. Antimicrobial potency of cumin and ginger has also been reported against different types of microorganisms (Arora and Kaur, 1999; Joe et al., 2009; Das et al., 2012; De Britto et al., 2012; Iram et al., 2012; Ismail et al., 2012; Mishra and Behal, 2012; Kota and Paladi, 2013; Revati et al., 2013; Shabaan et al., 2013; Virendra et al., 2013).

Although many different herbs and spices have been tested for their antimicrobial properties, fewer investigations on the synergistic/additive or antagonistic effects of spice extracts have been conducted. Combinations of extracts can also lead to additive or synergistic or antagonistic effects. Synergy, the interaction of compounds to create more profound microbial action, may be an important factor in using spices for antimicrobial actions. The additive effect is equal to the individual effects, whereas the antagonistic effect is less potent than the individual effects. Synergism often results from components of one spice aiding the other, improving the total efficacy. There are very few reports on the synergistic/antagonistic effects of spice extracts especially on food-spoilage microorganisms (Das et al., 2012; Kota and Paladi, 2013). The knowledge of synergistic/antagonistic antimicrobial effects of extracts of the spices of *Cuminum cyminum*, *Zingiber officinale* and *Allium sativum* can have vital implications in relation to the Indian culinary habits as most of these spices are used in blends in various Indian food preparations. Therefore, it is important to study the antimicrobial effects of these spices in their individual capacity as well as in combinations.

Materials and Methods

Spice samples and their extract preparation

Three most commonly used spices namely cumin (*Cuminum cyminum*), ginger (*Zingiber officinale*) and garlic (*Allium sativum*) were purchased from the local market. The spices were dried fruits of cumin, fresh rhizomes of ginger and fresh bulbs of garlic. The separable part (husk) of ginger and garlic was removed and the spices were washed with distilled water, air-dried and ground finely in a laboratory blender. For preparation of extracts, 25 g of ground and air-dried

spice material was shaken with 100 ml of 96% (w/v) ethanol at room temperature with continuous stirring for 4 days. The collected extracts were concentrated using rotary vacuum evaporator (Ika, Germany) and further the ethanol was evaporated to dryness using vacuum oven at 40°C. The extract was weighed and dissolved in ethanol to a concentration of 200 mg/ml and stored at refrigeration temperature in sterile vials for further experiments. The percent yield of the ethanolic extracts was calculated by the following formulae:

Percent yield = Weight of crude extract obtained in g x 100/total weight of raw spice in g (dwb).

Test microorganisms

The bacterial strains of *Bacillus subtilis* (MTCC 441, gram +ve), *Pseudomonas fluorescens* (MTCC 1749, gram +ve), *Salmonella* Typhi (ATCC 1311, gram -ve) and fungal strains of *Candida albicans* (MTCC 183, pathogenic) and *Rhizopus azygosporus* (MTCC 10195, non-pathogenic) were selected for the study. All the microbial cultures in their freeze dried form were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh (India). The freeze dried cultures of *Bacillus subtilis* (MTCC 441), *Salmonella* Typhi (MTCC 1311), *Pseudomonas fluorescens* (MTCC 1749) were grown on nutrient agar medium at 30°C for 24 h. Fungal cultures of *Candida albicans* (MTCC 183), *Rhizopus azygosporus* (MTCC 10195) were grown on potato dextrose agar (PDA) at 28°C for 7 days.

Preparation of inoculums

Bacterial strains were grown on nutrient agar plates and fungal strains were grown on potato dextrose agar plates. Bacterial inoculums were prepared from overnight grown cultures in peptone water and the turbidity was adjusted equivalent to 0.5 McFarland units (approximately 10² CFU/ml for bacteria and for fungi inoculums turbidity was equivalent to 10⁶ CFU/ml). The microorganism were inoculated into peptone water and incubated at 35± 2°C for 1-2 h (Manila et al., 2014).

Antimicrobial activity assay

Antimicrobial studies were carried out using agar well diffusion method. The petri-plates containing nutrient agar or potato dextrose agar were spread with inoculums with a sterile glass spreader. Wells were made at equal distance using a sterile stainless steel borer and each well was filled with 50 µl of the extract. Apart from using individual extracts of different

spices, the blended extracts of cumin + ginger, ginger + garlic and cumin+ ginger + garlic were also used to assess their synergistic or antagonistic antimicrobial effects, if any. The plates were incubated for a period of 24 h at 37°C in an incubator for bacterial growth and at 30°C for 28-48 h for fungal growth. Diameter (mm) of the clear inhibitory zone formed around the well was measured. Microorganisms showing a clear zone of more than 12 mm were considered to be inhibited (Arora and Kaur, 1999). Each experiment was performed in triplicate and mean values was taken.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that can inhibit the visible growth of a microorganism after overnight incubation. The MIC values of extracts were determined based on a micro broth dilution method in 96 multi- well microtiter plate. A volume of 100 µl test material in ethanol (a stock concentration of 200 mg/ml of crude extract) added in the first row of the plate. 50 µl of nutrient broth and 50 µl of saline solution were added to each well of plate. Serial dilutions were performed using a multichannel pipette such that each well had 100 µl of serially descending concentrations. 10 µl of resazurin indicator solution (prepared by dissolving a 270 mg resazurin tablet in 40 ml of sterile distilled water) was added in each well. Finally 10 µl of microbial suspension was added to each well to achieve a concentration of 5×10^6 CFU/ml. Each plate was wrapped with cling film to ensure that bacteria did not become dehydrated. Each plate had a column with streptomycin as positive control. The plates were prepared in triplicate and placed in an incubator at 37°C for 18 to 24 h. Any color changes from purple to pink or to colorless indicated growth of microbes. The lowest concentration at which no color change occurred was taken as the minimum inhibitory concentration value of extract (Manila *et al.*, 2014).

The synergistic/additive effect of various spice extracts was determined by calculating the fractional inhibitory indices according to the formula: $\sum FIC = FIC A + FIC B = [A]/MIC A + [B]/MIC B$. FIC A is the MIC of extract A in the combination/ MIC of extract A alone, and FIC B is the MIC of extract B in the combination/ MIC of extract B alone. The results were interpreted as synergy (FIC index < 0.5), addition ($0.5 \leq FIC \text{ index} \leq 1$), indifference ($1 < FIC \text{ index} \leq 4$) or antagonism (FIC index > 4) (Gutierrez *et al.*, 2009).

Results and Discussion

Spice extracts were prepared, filtered and evaporated as per standard procedures. Percent yield of cumin, ginger and garlic extracts was 1.19, 0.92 and 0.64 respectively on dry weight basis. The antimicrobial activity of individual extracts and their combinations against the selected microorganisms was assessed by presence or absence of inhibition zone. The zone of inhibition was estimated by agar well diffusion assay and the diameters of zones are as shown in table 1. The entire individual extracts and their different combinations showed broader antimicrobial activity as these were more or less inhibitory against bacteria as well as fungus *C. albicans*. However, absence of inhibition zone against *R. azygosporus* suggested that this fungus was resistant to the inhibitory activity of these spices. The diameter of inhibition zones of individual extracts of different spices ranged from 11.6 to 17.3 mm against bacteria and from 16.3 to 19.3 mm against fungus *C. albicans*. The agar well diffusion assay for antimicrobial activity yielded the inhibitory zones of 12.8 to 18.3 mm diameter for cumin, 11.5 to 16.3 mm diameter for ginger and 16.8 to 19.3 mm diameter for garlic extract indicating that garlic was the most effective spice in inhibiting the microbial growth. The combined extracts showed inhibition zones ranging from 12.3 to 19.6 mm in diameter against bacteria and 15.6 to 19.6 mm against fungus *R. azygosporus*. The combined extract of cumin and garlic was found to be most effective in inhibiting the microbial growth. Gram-ve bacteria and fungus were more prone to the inhibitory activity in comparison to gram +ve bacteria. Generally, gram -ve bacteria are supposed to be more resistant to antibiotics than gram +ve bacteria (Zaika *et al.*, 1983) but the present study revealed contradictory observations that gram-ve bacteria were more susceptible to inhibition by the crude spice extracts. The hydrophobicity of the spice extracts components and their broad spectrum of antibiotic compounds might be responsible for having inhibitory activity against gram -ve bacteria. As evident from the inhibition zones diameter values given in table 1, the individual extracts of all the three spices were most inhibitory against the growth of *C. albicans*. Differential antimicrobial activity of extracts against different microbes might be due to the presence of different phyto-compounds (Das *et al.*, 2012), which may include terpenoides, alkaloids and phenolic compounds (Hoult and Paya, 1996; Rios and Recio, 2005). Antimicrobial activity of alcoholic extracts of ginger has been reported against *B. subtilis* and *C. albicans* and agar well diffusion

Table 1. Antimicrobial activities as indicated by inhibition zones of selected spice extracts and their combinations against microorganisms

Spice extracts	Diameter of zones of inhibitions (mm) against microorganisms			
	<i>B. subtilus</i>	<i>P. fluroscens</i>	<i>S. Typhi</i>	<i>C. albicans</i>
Cu	12.8±0.5	15.5±1.0	13.8±0.2	18.3±1.0
Gi	11.6±1.0	13.4±0.7	11.5±0.3	16.3±0.7
Ga	16.8±0.5	17.1±0.7	17.3±0.5	19.3±0.5
CuGi	16.4±0.7	12.3±1.0	18.1±0.5	15.6±0.4
CuGa	17.4±0.7	17.8±1.0	19.0±0.3	19.6±0.2
GiGa	17.1±0.7	13.9±0.9	16.7±0.2	16.3±0.3
CuGiGa	15.6±1.0	12.6±0.2	13.0±0.4	18.0±0.4

Cu-cumin, Gi-ginger, Ga-garlic, CuGi- combined extracts of cumin and ginger, CuGa- combined extracts of cumin and garlic, GiGa- combined extracts of ginger and garlic, CuGiGa- combined extracts of cumin, ginger and garlic

Table 2. Minimum inhibitory concentration (mg/ml) of various spice extracts and their combinations

Spice extracts	Minimum inhibitory concentrations against selected Microorganisms			
	<i>B. subtilus</i>	<i>P. fluroscens</i>	<i>S. Typhi</i>	<i>C. albicans</i>
Cu	12.5±0.0	12.5±0.0	12.5±0.0	12.5±0.0
Gi	12.5±0.1	12.5±0.1	12.5±0.0	12.5±0.2
Ga	12.5±0.2	12.5±0.1	12.5±0.1	12.5±0.1
CuGi	6.3±0.0	6.7±0.0	6.3±0.0	6.7±0.2
CuGa	6.3±0.1	6.7±0.0	6.3±0.0	6.3±0.0
GiGa	6.3±0.0	3.8±0.1	3.8±0.1	6.7±0.0
CuGiGa	6.3±0.0	6.3±0.1	3.8±0.0	6.3±0.0

Cu-cumin, Gi-ginger, Ga-garlic, CuGi- combined extracts of cumin and ginger, CuGa- combined extracts of cumin and garlic, GiGa- combined extracts of ginger and garlic, CuGiGa- combined extracts of cumin, ginger and garlic.

method has shown inhibition zones from 15 to 35 mm diameter for ginger (Mishra and Behl, 2010). Antimicrobial effect of alcoholic extract of cumin against different microorganisms has been studied to have significant growth inhibition against *A. tumefaciens*, *B. subtilus*, *M. lutes*, *E. aerogenes* and *E. coli* but no growth inhibition against some fungi (De et al., 2003). Excellent to moderate Inhibitory effects of garlic extract at different concentrations against bacterial strains of *S. Typhi*, *E. coli*, and *S. aureus* has been studied (Joe et al., 2009; Iram et al., 2012). The antibacterial activity of garlic is reported due to the action of allicin or diallyl thiosulphinic acid or diallyl disulphate (Avato et al., 2000). It is postulated that the antibacterial and antifungal properties of garlic juice are due to the inhibition of succinic dehydrogenase via the inactivation of thiol group. The results of the present study are also in agreement in relation to the observation that there was no inhibition of the growth of fungus *R. azygosporus* by the cumin extract. Skrinjar and Nemet (2009) tested the antimicrobial activity of essential oils of garlic, cumin and ginger against the most common bacteria and fungi that contaminate food including

Listeria spp., *Staphylococcus*, *Salmonella* spp., *Escherichia* spp., *Pseudomonas* spp., *Aspergillus* spp., *Cladosporium* spp. and it was reported that garlic, cumin and ginger had very strong, medium and weak inhibitory effects respectively. The results of the present study concur with these observations as garlic had highest antimicrobial activity and cumin showed medium and ginger showed weak inhibitory effect.

Although various spice extracts showed different inhibitory effect against tested micro-organisms, similar value of minimum inhibitory concentration of 12.5 mg/ml was observed in case of each individual spice extract against all the tested micro-organisms (Table 2). This showed that the same concentration of various individual extracts was effective to different extents in inhibiting the growth of tested microorganisms. The MIC of combined extracts however fluctuated from 3.8 to 6.7 mg/ml and the most sensitive microbial species in relation to the MIC of combined extracts was *S. Typhi* for which a minimum concentration of 3.8 mg/ml of the combined extracts of ginger and garlic as well as ginger, garlic and cumin was inhibitory (Table 2). Minimum inhibitory

Table 3. Antimicrobial activities as indicated by fractional inhibitory concentration (mg dry weight ml⁻¹) of combined spice extracts against selected microorganisms

Combined extracts	<i>B. subtilis</i>	<i>P. fluorescens</i>	<i>S. Typhi</i>	<i>C. albicans</i>
CuGi	1.00	1.07	1.00	1.07
CuGa	1.00	1.00	1.00	1.00
GiGa	1.00	0.608	0.608	1.07
CuGiGa	1.00	1.00	0.608	1.00

concentration values ranging from 3.125 to 12.5 mg/ml has been reported by Mishra and Behl (2010) for ginger extract against *B. subtilis*, *S. aureus*, *E. coli*, *P. aeruginosa* and *P. chrysogenum*, *C. albicans*. The MIC of most of the combined extracts was reduced to almost half of the MIC of individual extracts. The fractional inhibitory concentrations (FIC) values of the combined extracts as given in table 3 showed that none of the combinations displayed synergistic effect (FIC index < 0.5) against any of the bacteria tested. However, all the combined extracts revealed additive effect ($0.5 \leq \text{FIC index} \leq 1$) against all the microbial species and this additive effect was more pronounced against *S. Typhi* and *P. fluorescens* particularly in case of combined extract of ginger and garlic and the combined extract of all the three spices (FIC value of 0.608). It is evident from the results that no combination showed antagonistic effect. The antimicrobial effect of combined extracts of different spices as studied by Witkowska *et al.* (2013) witnessed no synergistic effect of combinations. However, additive effect of combination of oregano and rosemary extracts was reported against *L. innocua* and *S. aureus*. The additive effect of these spices against tested microorganisms supports the use of these spices in combinations. The results of the study revealed that combined extracts were more effective as antimicrobials as the antimicrobial properties of herb and spice derived preparations depend not only on chemical composition but also on the lipophilic properties and water solubility, combinations of various compounds may have contributed to the observed additive effects (Lambert *et al.*, 2001; Gutierrez *et al.*, 2008). The multiple mode of action may include degradation of the cell wall, disruption of the cytoplasmic membrane, leakage of cellular components, alteration of fatty acid and phospholipids constituents, changes in the synthesis of DNA and RNA and destruction of protein translocation (Shan *et al.*, 2007). Hence, it is possible that combining spice extracts could lead to synergistic or additive inhibitory potential against both food spoilage and pathogenic microorganisms. Most studies attributed additive and synergism effects to phenolic and alcohol compounds. Generally compounds with similar structures exhibit

additive rather than synergistic effect (Lambert *et al.*, 2001; de Azeredo *et al.*, 2011; Bajpai *et al.*, 2012).

Conclusion

Based on observations of this study on antimicrobial activities of garlic, cumin and ginger, it can be concluded that these spices can be used as effective antimicrobial agents against both gram +ve and gram -ve bacteria as well as pathogenic fungi in individual as well as combined forms. Combinations of their extracts in can provide additive or synergistic inhibitory effects making them more effective as antimicrobial agents. Even though traditionally, spices are used as food preservatives and antiseptics, it is necessary to establish their antimicrobial properties by standardizing their concentrations in the combined extracts so that optimum inhibitory effect may be obtained. Further studies are required to investigate the mechanism of interaction of different phytochemicals from different spices and their mechanism of microbial growth inhibition. The knowledge on efficacy of combined extracts can be extended from culinary food applications to pharmacology and food chemistry.

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