COMPARATIVE STUDIES ON THE ANTIMICROBIAL ACTIVITY OF BLACK PEPPER (*PIPER NIGRUM*) AND TURMERIC (*CURCUMA LONGA*) EXTRACTS

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ABSTRACT: In the present study, a total of six extracts of two spices namely black pepper and turmeric in three solvents were evaluated for their antibacterial and antifungal activity. The antibacterial activity was measured by agar well diffusion method and antifungal activity by poisoned food technique. All the extracts showed antibacterial activity against all the test bacterial isolates. Aqueous extracts of black pepper did not exhibit antibacterial activity against *B. subtilis*. In aqueous extract, black pepper and turmeric showed good inhibitory activity against *Staphylococcus aureus* with zone of inhibition 25mm to 30mm and 26mm to 28mm respectively. In ethanolic extract, black pepper extract showed antibacterial activity against all test bacteria with zone of inhibition ranged between 15mm and 22mm while turmeric showed activity with zone of inhibition ranged between 13mm and 24mm. In methanolic extract, the diameter of zone of inhibition ranged between 12mm and 28mm in black pepper and 13mm and 22mm in turmeric. In case of antifungal activity, only turmeric ethanolic extract showed activity only against *Rhizopus stolonifer* and *Mucor* sp. with percent mycelial growth inhibition ranged between 25% and 30%. Based on this finding, these extracts may be an alternative to chemical preservatives and used as natural antimicrobial preservatives to reclaim the shelf-life of food.

Key words: Agar well diffusion, antibacterial and antifungal activity, black pepper, extracts, spices

INTRODUCTION

Many plant derived products such as spices, fruit preparations, vegetable preparations or extracts have been used for centuries for the preservation and extension of the shelf life of foods (Chattopadhyay and Bhattacharyya, 2007). Spices have been defined as plant substances from indigenous or exotic origin, aromatic or with strong taste, used to enhance the taste of foods. Spices include leaves (coriander, mint), flower (clove), bulbs (garlic, onion), fruits (red chilli, black pepper), stem (cinnamon), rhizomes (ginger, turmeric) and other plant parts (Shelef, 1983). Prevention of pathogenic and spoilage microorganisms in food is usually achieved by using chemical preservatives but they are responsible for many carcinogenic and teratogenic attributes as well as residual toxicity and with growing concern of microbial resistance towards conventional preservatives, consumers tend to be suspicious of chemical additives and thus the exploration of naturally occurring antimicrobial for food preservations receives increasing attention (Schuenzel and Harrison, 2000). The objective of this study was to evaluate the antimicrobial activity of two plant extracts against food associated bacteria and fungi.

MATERIALS AND METHODS

Test microorganisms

Ten food-associated bacteria (7 Gram-positive and 3 Gram-negative) isolates (*Bacillus subtilis* I, *B. megaterium* I, *B. sphaericus, B. polymyxa, Staphylococcus aureus* I, *S. aureus* II, *S. aureus* III (Gram-positive), *Escherichia coli* I, *E. coli* II and *E. coli* III (Gram-negative) and eleven molds (*Aspergillus luchuensis* I, *A. luchuensis* II, *A. flavus* I, *A. flavus* II, *Penicillium oxalicum* I, *Rhizopus stolonifer* I, *R. stolonifer* II, *Scopulariopsis* sp. I, *Scopulariopsis* sp. II, *Mucor* sp. I and *Mucor* sp. II) were isolated and identified from bakery products and pickles and screened against plant extracts.

Preparation of bacterial inoculum

The density of selected food-associated bacteria was adjusted equal to that of the 0.5 McFarland standard (1.5×10^8 CFU/ml) by adding sterile distilled water. McFarland standards are used as a reference to adjust the turbidity of microbial suspension so that the number of microorganisms will be within a given range. For the preparation of the 0.5 McFarland standard, 0.05ml of barium chloride ($BaCl_2$) (1.17% w/v $BaCl_2.2H_2O$) was added to 9.95 ml of 0.18M H_2SO_4 (1.0% w/v) with constant stirring. The McFarland standard tube was tightly sealed to prevent loss by evaporation and stored for up to 6 months. To aid comparison the test and standard were compared against a white background with a contrasting black line (Andrews, 2001).

Preparation of fungal inoculum

The stock suspensions of eleven food-associated fungal isolates were standardized to 10^6spores/ml by spectrophotometrically at 530nm and were adjusted to 80 to 85% transmittance. The fungal inoculum (10^6spores/ml) was also determined by plate count on PDA followed by incubation at 25^0C for 7 days and observations made for visible growth of fungi at regular interval during the incubation period (Rasooli and Abyanek, 2004).

Evaluation of plants for their antimicrobial activity

Collection of plant parts

Plant parts of black pepper (*Piper nigrum*) and turmeric (*Curcuma longa*) were collected from localities of Kurukshetra, Haryana and evaluated for their antimicrobial activity against ten food-associated bacteria and eleven fungi. These plant parts were selected on the basis of their use in folk medicine and as alternative system of health care and as food preservatives (Arora and Kaur, 1999; Indu, et al., 2006; Chattopadhyay and Bhattacharyya, 2007; Parekh and Chanda, 2007).

Protocol for phytochemical extraction

For extraction, the freshly collected plant parts were thoroughly washed with tap water followed by sterile distilled water. The material was dried in an oven at 50°C for 48 hrs followed by grinding in to a fine powder. The powdered material was stored in air tight jars in refrigerator at 4°C (Lin, et al., 2004). Three extractants i. e., water, ethanol (95%) and methanol (95%) were used for the phytochemical extraction of two plant parts. A total of 6 extracts (aqueous, ethanolic and methanolic) were prepared from two plant parts.

For extraction with water, 25 g of powdered plant material was dissolved in enough sterilized distilled water to make 100ml of aqueous extract (25% w/v). The mixture was kept undisturbed at room temperature for 24 hrs in a sterile flask covered with aluminum foil to avoid evaporation and subjected to filtration through sterilized Whatman no.1 filter paper. After filtration, the extract was evaporated in water bath until 25 ml extract was left in the container. For extraction with ethanol, twenty five gram of powdered plant material was dissolved in enough ethanol to make 100ml of ethanolic extract (25% w/v). The extraction procedure followed was the same as used for aqueous extract. For extraction with methanol, twenty five gram of powdered plant material was dissolved in enough methanol to make 100ml of methanolic extract (25% w/v). The extraction procedure followed was the same as used for aqueous extract. Extracts thus obtained were immediately evaluated for antibacterial and antifungal activities (Barreto, et al., 2002).

Agar well diffusion method

The antibacterial activity of 6 crude extracts (aqueous, ethanolic and methanolic) of 2 plant parts against all the ten food-associated bacterial isolates was evaluated by using agar well diffusion method (Ahmad and Beg, 2001; Srinivasan, et al., 2001). PCA plates were inoculated with 100µl of standardized inoculum ($1.5x10^8$ CFU/ml) of each bacterium (in triplicates) and spread with sterile swabs. Wells or cups of 8 mm size were made with sterile borer into agar plates containing the bacterial inoculum and the lower portion was sealed with a little molten agar medium. 100µl volume of the plant extract was poured into a well of inoculated plates. Sterilized distilled water or solvent (ethanol/methanol) was used as a negative control which was introduced into a well instead of plant extract. Acetic acid was used as a positive control which was introduced into the well instead of plant extract. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar (Rios, et al., 1988). After incubation for 24 hrs at 37°C, the plates were observed. If antibacterial activity was present on the plates, it was indicated by an inhibition zone surrounding the well containing the plant extract. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded if the zone of inhibition was greater than 8 mm (Hammer, et al., 1999). The antibacterial activity results were expressed in term of the diameter of zone of inhibition and <9mm zone was considered as inactive; 9-12mm as partially active; while13-18mm as active and >18mm as very active (Junior and Zanil, 2000). The mean and standard deviation of the diameter of inhibition zones were calculated.

Poisoned food technique

The antifungal activity of 6 plant extracts was evaluated against food-associated fungi by using poisoned food technique. In poisoned food technique, all the food-associated fungi were inoculated on PDA plates in triplicates and incubated for 25^0C for 3 to 7 days, to obtain young, actively growing colonies of molds. 100µl of plant extract was mixed with 15ml of cooled (45^0C) molten PDA medium, poured on to the plates and allowed to solidify at room temperature for thirty minutes. A mycelial disc 6mm diameter, cut out from periphery of 3 to 7 day old cultures, was aseptically inoculated onto the agar plates containing the plant extract. PDA plates with 100µl of solvent and acetic acid were used as negative and positive control respectively (McCutcheon, et al., 1994). The inoculated plates were incubated at 25^0C and colony diameter was measured and recorded after 7 days. Percent mycelial growth inhibition was calculated as given below:

$$\text{Percent mycelial growth inhibition} = \frac{\text{Diameter of fungal colony (mean) in control} - \text{diameter of fungal colony (mean) with plant extract}}{\text{Diameter of fungal colony (mean) in control}} \times 100$$

RESULTS AND DISCUSSION

There has been an increasing consumer demand for foods free or with low, if any, added synthetic preservatives because synthetic preservatives could be toxic to humans (Bedin, et al., 1999). Concomitantly, consumers have also demanded for wholesome and safe food with long shelf lives. These requirements are often contradictory and have put pressure on the food industry for progressive removal of chemical preservatives and adoption of natural alternatives to obtain its goals concerning safe food with long shelf lives (Brull and Coote, 1999).

Spices and herbs nave been used for thousand of the centuries by many cultures to enhance the flavour and aroma of food. Early culture also reorganized the values of using spices and herbs in preventing foods and for their medical values. Spices in the past decade confirm that the growth of both Gram-positive and Gram-negative foodborne bacteria, yeasts and molds can be inhibited by garlic, onion, cinnamon, clove, thyme, sage and other spices. Although, the primary purpose of spices is to impart flavour and piquancy to food, the medicinal, antimicrobial and antioxidant properties of spices have also been exploited (Souza, et al., 2005). The antimicrobial activity of spices is documented and interest continues to the present (Uraih, 2004).

The growing concern about food safety has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria. Spices are one of the most commonly used natural antimicrobial agents in foods and have been used traditionally for thousands of years by many cultures for preserving foods and as food additives to enhance aroma and flavour (Souza, et al., 2005).

The present research work deals with the antimicrobial evaluation of plants (black pepper and turmeric). Each plant material was dried and grinded to a fine powder before subjecting to crude phytochemical extraction. The active phytochemical components would be expected to be more concentrated in dry preparation than in fresh plant material (Romero, et al., 2005).

Naturally occurring water-soluble components in most plant materials include various anionic components such as thiocynate, nitrate, chlorides and sulphates, starches and tannins, saponins, terpenoids, polypeptides and lectins (Darout, et al., 2000). Phytochemicals having solubility in ethanol include tannins, polyphenols, polyacetylenes, flavonol, sterols and alkaloids (Ivanovska, et al., 1996). Cowan, (1999) examined a variety of extractants for their ability to solubilize antibacterials from plants as well as other factors such as their relative ranking as biohazards and the ease of removal of solvent from the fraction and ranked them in the order: methylene dichloride > methanol > ethanol > water. Accordingly, in the present study, three solvents namely water, ethanol and methanol were selected for the plant extraction. In the present study, the black pepper and turmeric extracts exhibited antibacterial activity in all the three kinds of solvents. Aqueous, ethanolic and methanolic extracts of black pepper exhibited activity against *B. megaterium, B. sphaericus, B. polymyxa, S. aureus* and *E. coli*, this substantiate the findings of Ali, et al., (2007), who had been reported antibacterial activity of water, petroleum ether, ethyl acetate, ethanolic and methanolic black pepper extracts against *B. megaterium, B. subtilis, S. aureus* and *E. coli*. According to Harold (2004), the antimicrobial activity of black pepper is due to the presence of essential oil (3%), whose aroma is dominated by monoterpenes hydrocarbons: sabinene, β-pinene and limonene. Furthermore, terpinene, α-pinene, myrcene, and monoterpene derivatives like borneol, carvone, carvacrol, 1, 8-cineol and linalool are also present. The mechanism of action of terpene is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Ahmed, et al., 1993).

A perusal of the data in the table 1 reveals that all the three types of turmeric extracts possessed activity against all the food associated bacteria. The ethanolic extract being strongly active against *E. coli* isolates while aqueous extracts strongly active against *S. aureus* isolates (25mm-30mm). Chandrana, et al., (2005) who studied antimicrobial activity of turmeric reported that it was effective against *E. coli, B. subtilis* and *S. aureus* and suggested that the activity is due to the presence of curcuminoid, a phenolic compound. Gur, et al., (2006) who reported that the ethanolic extract of turmeric was effective in extraction of antimicrobially active substances as compared to water and hexane. Negi, et al., (1999) demonstrated that turmerone and curlone components of turmeric possess excellent antibacterial action against a wide range of microbes such as *B. cereus, B. coagulans, B. subtilis, S. aureus. E. coli* and *P. aeruginosa*. The antimicrobial property of turmeric has been attributed to the presence of essential oil, an alkaloid, curcumins and other curcuminoids, turmeric oil, turmerol and veleric acid (Cikrikci, et al., 2008).

Table 1. Antibacterial activity of two plant extracts in three solvents against food-associated bacteria by agar well diffusion method

Solvent	Plant Name	Diameter of inhibition zone (mm)[a]									
		Bs I	Bm I	Bsph	Bp	Sa I	Sa II	Sa III	Ec I	Ec II	Ec III
Aqueous	Black pepper	-	18±0.57[b]	12±0.37	13±0.57	30±0.81	25±0.57	28±0.81	13±0.37	11±0.37	12±0.57
	Turmeric	17±0.37	15±0.57	16±0.81	15±0.81	28±0.57	27±057	26±0.37	18±0.81	17±0.37	17±0.37
Ethanolic	Black pepper	15±0.57	20±0.81	18±0.37	18±0.81	20±0.57	18±0.81	19±0.57	22±0.57	20±0.81	21±0.81
	Turmeric	15±0.81	14±0.57	13±0.57	12±0.57	20±0.37	17±0.57	18±0.57	24±0.81	23±0.37	22±0.37
Methanolic	Black pepper	19±0.57	20±0.37	18±0.37	17±0.37	12±0.57	10±0.57	12±0.37	22±0.37	21±0.57	20±0.81
	Turmeric	14±0.57	12±0.81	13±0.57	12±0.57	20±0.57	19±0.57	18±0.57	28±0.57	27±0.57	26±0.81
Acetic acid (Positive control)		22±0.81[b]	22±0.81	20±0.57	20±0.57	17±0.37	17±0.37	16±0.37	22±0.81	21±0.81	20±0.81
Distilled water (Negative control)		-	-	-	-	-	-	-	-	-	-

The mechanism of antibacterial action of spices and derivatives is not yet clear (Lanciotti, et al., 2004). Hypothesis have been proposed different workers (Odhav, et al., 2002) which involve: hydrophobic and hydrogen bonding of phenolic compounds to membrane proteins, followed by partition in the lipid bilayer; perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzymes; membrane disruption; destruction of electrons transport systems and cell wall perturbation.

Turmeric ethanolic extract showed inhibitory activity active against only two molds namely *R. stolonifer* and *Mucor* sp. (Table 2). Turmeric has also been shown to induct predominant antifungal activity (Arora, 1999). However, there are reports of occurrence of antifungal activity against other molds such as *Aspergillus niger* and *Penicillium digitatum* (Kapoor, 1997) and *A. niger, A. flavus, P. javanium, Curvularia oryzae* and *Trichophyton mentagrophytes* (Arora, 1999).

The fungistatic or fungicidal effect of spices is due to the inhibitory action of natural products and the mechanisms involved are cytoplasm granulation, cytoplasmic membrane rupture and inactivation and/or inhibition of intracellular and extracellular enzymes. These biological events could take place separately or concomitantly culminating with mycelium germination inhibition (Cowan, 1999) and it is also reported that plant lytic enzyme act in the fungal cell wall causing breakage of β-1,3 glycan, β-1,6, glycan and chitin polymer (Brull and Coote, 1999).

These extracts did not possess antifungal activity except turmeric ethanolic extract against the food-associated molds. There may several seasons for the lack of antimicrobial activity in their plants, either the plant part used or the type of extraction might have resulted in the nil activity in this study (Arora and Kaur, 1999) or the time of collection of herbal material and climate, which might, in turn, affect the amount of active constituents in the plant material (Parekh and Chanda, 2007).

The antimicrobial action of the aqueous extracts could be ascribed to the anionic components such as thiocyanate, nitrate, chlorides and sulphates besides other water soluble components which are naturally occurring in the plant material (Darout, et al., 2000). The ethanol extraction of herbs and spices was better because ethanol is an organic solvent and dissolves more organic compounds, resulting in the liberation of the greater amounts of active antimicrobial components (Cowan, 1999). The water was found to be least effective in extracting the active antimicrobial component/s present in the spices in the present study. The differences in the sensitivity of food-associated microorganisms may be due to the differences in concentrations, methods of extraction used in each study (Kumar, et al., 1997) and the little diffusion properties of these extracts in the agar and soil composition and water availability (Romero, et al., 2005).

Table 2. Antifungal activity of two plant extracts in three solvents against food-associate fungi by poisoned food technique.

Solvent	Aqueous plant extract	Percent mycelial growth inhibition										
		Alu I	Alu II	Afl I	Afl II	Pox I	Rst I	Rst II	Mu I	Mu II	Sco I	Sco II
Aqueous	Black pepper	-	-	-	-	-	-	-	-	-	-	-
	Turmeric	-	-	-	-	-	-	-	-	-	-	-
Ethanolic	Black pepper	-	-	-	-	-	-	-	-	-	-	-
	Turmeric	-	-	-	-	-	25	30	25	25	-	-
Methanolic	Black pepper	-	-	-	-	-	-	-	-	-	-	-
	Turmeric	-	-	-	-	-	-	-	-	-	-	-
Acetic acid (Positive control)		100	100	100	100	66.6	100	100	100	100	60	60
Distilled water (Negative control)		-	-	-	-	-	-	-	-	-	-	-

- No activity

Abbreviations

Alu I- *Aspergillus luchuensis* I, Alu II- *Aspergillus luchuensis* II, Afl I- *Aspergillus flavus* I, Afl II-*Aspergillus flavus* II, Pox I- *Penicillium oxalicum* Rst I-*Rhizopus stolonifer* I, Rst II-*Rhizopus stolonifer* II, Mc I-*Mucor* sp. I, Mc II-*Mucor* sp. II, Sco I- *Scopulariopsis* sp. I, Sco II- *Scopulariopsis* sp.II.

CONCLUSION

It may be concluded from the present studies that both the extracts can be used as a potential source of natural antimicrobial compound which if applied to food products. Further research is required for the identification of bioactive molecule present in the two extracts and *in vivo* efficacy against food spoilage microorganisms before it is used for commercialization in the form of food preservatives, additives and nutraceutical foods.

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