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Antimicrobial Activity of Ethanolic Extracts of *Syzygium aromaticum* and *Allium sativum* Against Food Associated Bacteria and Fungi

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Abstract

The successful control of food spoilage microorganisms require the use of indigenous antimicrobials in foods including certain botanical compounds that have been historically used for flavour enhancement as well as preservation. The present study was designed to evaluate the *in vitro* antimicrobial activity of ethanolic extracts of *Syzygium aromaticum* (clove) and *Allium sativum* (garlic) against Gram-positive and Gram-negative food associated bacteria (*Bacillus subtilis, B. megaterium, B. polymyxa, B. sphaericus, Staphylococcus aureus* and *Escherichia coli*) and molds (*Penicillium oxalicum, Aspergillus flavus, A. luchuensis, Rhizopus stolonifer, Scopulariopsis* sp. and *Mucor* sp.) assayed by agar well diffusion method and poisoned food technique, respectively. Clove extract showed better antimicrobial activity than the garlic extract. The zone of inhibition in clove ethanolic extract against all the food associated bacteria was in the range of 25mm to 32mm and in molds the percent mycelial growth inhibition ranged from 70% to 100%. The growth inhibition zone in garlic ethanolic extract against bacteria was in the range of 20mm to 31mm and in molds the percent mycelial growth inhibition ranged between 20% and 50%. The clove ethanolic extract exhibited the maximum zone of inhibition against *E. coli* whereas garlic ethanolic extract showed maximum activity against *B. subtilis.* Both the extracts exhibited maximum percent mycelial growth inhibition against *R. stolonifer*. However garlic extract was not effective against *P. oxalicum.* The MIC values of clove ethanolic extract for different bacterial isolates ranged from 5.0mg/ml to 20mg/ml and 10 mg/ml to 20mg/ml against molds. The MIC values of garlic ethanolic extract for different bacterial and fungal isolates ranged from 10 mg/ml to 20mg/ml. The value of MBC and MFC equaled the MIC. Based on this finding, it may be suggested that these extracts may be used as natural antimicrobial additives to reclaim the shelf-life of foods.

Key words: Antimicrobial activity, food associated microorganisms, clove, garlic, MIC.

Introduction

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 (Nychas, 1995). 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), buds (clove), bulbs (garlic, onion), fruits (red chilli, black pepper), stem (cinnamon), rhizomes (ginger) and other plant parts (Shelef, 1983, Arora and Kaur, 1999).

Garlic (*Allium sativum*) is a common spice used for flavouring and has been traditionally popular with strong folkloric awareness. It is the edible bulb of lily family, *Liliaceae*. It contains aromatic sulphur based compounds, which contribute to the characterstics odour and taste. Antimicrobial activity of garlic is attributed to its key component allicin, which is a volatile molecule, gives garlic its characterstic odour. Allicin is unstable; once it is generated it readily decomposes to produce diallyl sulphide, dialyl disulphide, diallyl trisulphide, allyl methyl trisulphide, dithiins and ajoene (Jabar and Al-Mossawi, 2007).

Clove (*Syzygium aromaticum*) constitutes one of the major spices. Cloves are dried unopened floral buds of an evergreen tree, *Syzygium aromaticum* belonging to the family *Myrtaceae* (Shyamala *et al.*, 2003). Clove is used as flavouring agent and as spice for scenting, chewing tobacco. It is aromatic, stimulant & carminative, used for dyspepsia and gastric irritations. Clove buds and their essential oils have been known to possess various antimicrobial and antioxidant properties (Fu *et al.*, 2007). GC-MS analysis of the clove oil extract has shown eugenol acetate, eugenol and caryo-phyllene as the major constituents, the latter two are known to possess antibacterial and antifungal properties (Nassar *et al.*, 2007; Ayoola *et al.*, 2008). The objectives of this study were to evaluate the antibacterial and antifungal activity of ethanolic extracts of clove and garlic against six food-associated bacteria and six fungi.

Materials and Methods

Collection of plants

Two fresh plant parts including bud of clove (*Syzygium aromaticum*) and bulb of garlic (*Allium sativum*) were collected from localities of Kurukshetra, Haryana and evaluated for their antimicrobial activity against six food-associated bacteria and six fungi.

Test microorganisms and standardization of inoculum

The test bacteria namely *Bacillus subtilis, B. megaterium, B. sphaericus, B. polymyxa, Staphylococcus aureus* and *Escherichia coli* and fungi *Penicillium oxalicum, Aspergillus flavus, A. luchuensis, Rhizopus stolonifer, Scopulariopsis* sp., and *Mucor* sp. were isolated from bakery products such as breads, cakes, pastries, patties and buns collected from local market of Kurukshetra, Haryana, India. The density of six 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). The stock suspensions of six 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 (Florl *et al.*, 2003; Rasooli and Abyanek, 2004).

Phytochemical extraction

Drying

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 (Lin and Lineback, 1990).

Preparation of ethanolic plant extracts

An extract is a mixture of phytochemicals from any plant which is obtained by extraction of specific parts of the plant (Loew, 1997). Solvent, ethanol (95%) was used for the phytochemical extraction of various plant parts. For extraction with ethanol, 25 g of powdered plant material was dissolved in enough sterilized ethanol to make 100ml of ethanol 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. Ethanolic extracts thus obtained were immediately evaluated for antibacterial using agar well diffusion method and antifungal activities using poisoned food technique (Chen *et al.,* 1987, Barreto *et al.,* 2002).

Agar well diffusion method

The antibacterial activity of two crude ethanolic extracts of clove and garlic plant parts against six food-associated bacteria was evaluated by using agar well diffusion method (Ahmad and Beg, 2001, Srinivasan *et al.,* 2001). Plate count agar (PCA) plates were inoculated with 100µl of standardized inoculum ($1.5x10^8$ CFU/ml) of each selected 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. Chemical preservative, acetic acid was used as a positive control which was introduced into a well instead of plant extract. Solvent, ethanol was used as a negative control which was introduced into a 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^{o}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; while 13-18mm 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 plant extracts was evaluated against food-associated fungi by using poisoned food technique. In poisoned food technique, all the six food-associated fungi were inoculated on Potato dextrose agar (PDA) plates 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 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 acetic acid were used as positive control. PDA plates with 100µl of ethanol were used as negative control (Georgii and Korting, 1991, 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:

$$\% \text{ mycelial growth inhibition} = \frac{\text{Mean dia. of fungal colony in control - mean dia. of fungal colony in plant extract}}{\text{Mean diameter of fungal colony in control}} \times 100$$

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of clove and garlic ethanolic extracts against food-associated bacteria

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation (Andrews, 2001, NCCLS, 2002, Thongson *et al.*, 2004). MIC and MBC of clove and garlic ethanolic extracts were determined by macrodilution agar and broth methods (Andrews, 2001, NCCLS, 2002). The MIC and MFC were determined following the methodology of Florl *et al.* (2003), Rasooli and Abyanek (2004) and Irkin and Korukluoglu (2007).

Macrodilution agar method

In the macrodilution agar method, a two-fold serial dilution of the clove and garlic ethanolic extracts were prepared in sterile distilled water to achieve a decreasing concentration ranging from 160 to 1.25mg/ml in eight sterile tubes labeled 1 to 8. Sterile cork borer of 8.0mm diameter was used to bore well in the presolidified Mueller Hinton agar (MHA) plates and 100µl volume of each dilution was added aseptically into the wells made in MHA plates in triplicate that had food-associated bacteria seeded with the standardized inoculum (1.5 X 10^8 CFU/ ml). 100µl ethanol introduced into the well in place of plant extract was used as control. All the test plates were incubated at 37°C and were observed for the growth after 24 hrs. The lowest concentration of an extract showing a clear zone of inhibition was considered as the MIC.

Macrodilution broth method

In the macrodilution broth method, a two-fold serial dilution of the clove and garlic ethanolic extracts were prepared in sterile Mueller-Hinton broth to achieve a decreasing concentration ranging from 160 to 1.25mg/ml in eight sterile tubes labeled 1 to 8. Each dilution was seeded with 100µl of the standardized bacterial inoculum (1.5 X 10^8CFU/ml). The inoculated culture tubes were incubated at 37°C for 18 to 24 hrs. A set of tubes containing only seeded broth (i.e. without plant extract) was kept as control. The lower concentration that did not permit any visible growth when compared with the control was considered as the MIC.

The minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent that will prevent the growth of an organism after subculture on to antibiotic-free media. To determine the MBC, a 100µl aliquot from the tube showing MIC was placed on MHA plate antibiotic free and was spread over the plate. After incubation at 37^0C for 24hrs, the plates were examined for the growth of a bacterium to determine the concentration of the extract at which 99.9% killing of food-associated bacterial isolates was achieved.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of clove and garlic ethanolic extracts against food-associated fungi

Five ml of clove and garlic ethanolic extracts at different concentrations i.e. from 160 to 1.25mg/ml were taken in

to the sterile empty tubes and 1 ml of standardized fungal inoculum (10^6spores/ml) was added into the extracts and mixed. The stock suspensions of six fungal isolates were standardized spectrophotometrically at 530nm and were adjusted to 80 to 85% transmittance. The fungal inoculum (10^6spores/ml) was determined by plate count on PDA followed by addition of 1ml of both extract and fungal inoculum was added into the 5ml of sterile PDB in the tubes followed by incubation at 25^0C for 15 days and observations made for visible growth of fungi at regular interval during the incubation period. In control tubes, 1ml each of the extract and fungal inoculum were added into the 5ml of ethanol. The highest dilution (lowest concentration) showing no visible growth was regarded as minimum inhibitory concentration (MIC). 100µl aliquot from the tubes showing no growth were subcultured on PDA plates and inoculated PDA plates incubated at 25^0C for 5 days and observed for the development of the colonies to determine if the inhibition was reversible or permanent. Minimum fungicidal concentration (MFC) was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates (Florl *et al.*, 2003, Rasooli and Abyanek, 2004, Irkin and Korukluoglu, 2007).

Results and Discussion

The growing concern about food safety has recently led to the development of natural antimicrobials to control food borne and spoilage microorganisms. 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 (Nevas *et al.*, 2004, Souza *et al.*, 2005).

In the present investigation, the ethanolic extracts of clove and garlic showed inhibitory activity against all the six food associated bacteria in which the diameter of zone of growth inhibition varied between 25 and 32mm (in clove) and 20 and 31mm (in garlic) (Table 1). The clove ethnaolic extract showed highest diameter of zone of inhibition of 32mm against *E. coli* followed by *S. aureus* (31mm) and B. *subtilis* (30mm). The clove ethanolic extract showed similar zone of inhibition of 28 mm in diameter against *B. megaterium* and *B. sphaericus*. The minimum inhibitory activity was recorded against *B. polymyxa*. Our results substantiate the findings of Sulieman *et al*. (2007) who demonstrated the antibacterial activity of clove ethanolic extract against *E. coli*, *S. aureus* and *B. subtilis* and found that the highest antibacterial activity was against *E. coli*. The antibacterial activity of clove is attributed to eugenol (2 methoxy-4 allyl-phenol) (Gupta *et al.*, 2008). High tannin content (10-19%) in clove also provides additional antimicrobial activity (Namasombat and Lohasupthawee, 2005).

Table 1. Antibacterial activity of clove and garlic ethanolic extracts against food-associated bacteria by agar well diffusion method**.**

Ethanolic plant extract	Diameter of inhibition zone (mm[a])					
	Bs	Bm	Bsph	Bp	Sa	Ec
Clove	30±0.57	28±0.57	28±0.37	25±0.81	31±0.57	32±0.57
Garlic	31±0.81	20±0.81	20±0.81	21±0.57	30±0.37	30±0.57
Acetic acid (Positive control)	22±0.81[b]	22±0.81	20±0.57	20±0.57	17±0.37	22±0.81
Ethanol (Negative control)	-	-	-	-	-	-

- No activity; [a]-Values, including diameter of well (8mm), are means of the three replicate; [b] ± Standard deviation

Abbreviations

Bs - *Bacillus subtilis*, Bm - *B. megaterium*, Bsph-*B. sphaericus*, Bp-*B. polymyxa*, Sa - *Staphylococcus aureus* and Ec-*Escherichia coli*.

The garlic ethanolic extract demonstrated antibacterial activity against all the food associated bacteria with zone of growth inhibition ranging from 20mm to 31mm. The maximum zone of inhibition was showed against *B. subtilis* (31mm) followed by *S. aureus* and *E. coli* (30mm) and *S. aureus* (28mm). The zone of inhibition of 21mm was observed against *B. polymyxa*. The minimum diameter of zone of growth inhibition was recorded against *B. megaterium* and *B. sphaericus* (20mm). Garlic ethanolic extract showed inhibitory activity against all the tested *Bacillus* spp., *S. aureus* and *E. coli*. The antimicrobial activity of garlic has earlier been reported against *S. aureus, E. coli* and *Klebsiella pneumoniae* (Jabar and Mossani, 2007) and *E. coli* and *S. aureus* (Vuddhakul *et al*., 2007). Shelef (1983) reported that allicin, the essential oil substance isolated from garlic, inhibited bacteria in culture media and also discovered that most of the antimicrobial substances were phenol compounds such as eugenol, thymol and carvacol.

The ethanolic extract of clove was effective in terms of percent mycelial growth inhibition (70 to 100%) and garlic extract (20 to 50 %) (Table 2). The clove ethanolic extract showed excellent antifungal activity against *Rhizopus stolonifer* with complete mycelial growth inhibition (100%) followed by *Aspergillus luchuensis, A. flavus*, *Mucor* sp. (90%), *Scopulariopsis* sp. (75%) and minimum inhibition against *P. oxalicum* (70%). In the present invstigation, ethanolic extract of clove was found highly active against *Scopulariopsis* sp., *A. luchuensis, A. flavus, P. oxalicum, R. stolonifer* and *Mucor* sp. Several workers (Meena and Sethi, 1994, Arora and Kaur, 1999) have earlier reported that clove ethanolic extract showed antimycotic activity against fungal genera such as *Aspergillus*, *Penicillium, Rhizopus, Cladosporium* and *Saccharomyces* which is in hormony with the present study. This activity may be due to the presence of eugenol and caryophyllene.

The ethanolic extract of garlic exhibited partial activity against the two isolates each of *R. stolonifer* (50%), *Mucor* sp. (40%), *A. luchuensis* (30%), *A. flavus* (30%) and *Scopulariopsis* sp. (20%) but lacked in inhibitory activity against *P. oxalicum*. Both extracts possessed good antimicrobial activity against food associated bacteria and fungi. However, the antimicrobial activity was better in clove extract than garlic against all the test microorganisms. The inhibitory activity of *Allium* vegetable extracts against molds have been reported by numerous authors (Irkin and Korukluoglu, 2007, Mahmoudabadi and Nasery, 2009). Allicin, thiosulfonate and other compounds showed fungistatic activity against *Aspergillus* spp. such as *A. flavus, A. fumigatus, A. terreus* and *P. chrysogenum* (Harris *et al.*, 2001). Several studies have reported that garlic extract can inhibit the growth of bacteria, fungi, viruses in culture media and food systems and it has been shown to posses insecticidal, antiparasitic and antitumour properties (Kumar and Berwal, 1998). Several ajoene compounds, derivative of allicin, obtained from garlic with ethanol extraction has been found to be very inhibitory against *A. niger* and *Candida albicans* (Singh *et al.*, 1990, Neilsen and Rios, 2000, Irkin and Korukluoglu, 2007). Yashida *et al.* (1987) reported that ajoene compound from garlic have stronger antifungal agent than allicin. The MIC values of clove extract for different spoilage bacteria ranged from 5.0 to 20mg/ml (Table 3) and 10 to 20 mg/ml against molds (Table 4). The MIC values of garlic extract for different food associated bacteria and fungi ranged from 10mg/ml to 20mg/ml (Tables 5 and 6). The values of MBC and MFC were found to be equal to the MIC. The values of MBC and MFC were found to be equal to the MIC. Thus suggesting that evaluation of MIC is sufficient for measuring bactericidal and fungicidal activity. Natrajan *et al.* (2003) have also reported similar results.

Table 2. Antifungal activity of clove and garlic ethanolic extracts against food-associated fungi by poisoned food technique.

Ethanolic plant extract	Percent mycelial growth inhibition

	Alu	Afl	Pox	Rst	Mu	Sco
Clove	90	90	70	100	90	75
Garlic	30	30	-	50	40	20
Acetic acid (Positive control)	100	100	66.6	100	100	60
Ethanol (Negative control)	-	-	-	-	-	-

- No activity

Abbreviations

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

Table 3. Minimum inhibitory concentration (MIC) of clove ethanolic extract against food-associated bacteria on Mueller Hinton agar medium using macrodilution agar method.

Food associated bacteria	Concentration of clove ethanolic extract (mg/ml)								
	1.25	2.5	5.0	10.0	20.0	40.0	80.0	160.0	MIC
Bacillus subtilis	+	+	-	-	-	-	-	-	5.0
B. megaterium	+	+	+	+	-	-	-	-	20.0
B. sphaericus	+	+	-	-	-	-	-	-	5.0
B. polymyxa	+	+	-	-	-	-	-	-	5.0
Staphylococcus aureus	+	+	-	-	-	-	-	-	5.0
Escherichia coli	+	+	+	-	-	-	-	-	10.0

+ Growth; - No growth

Table 4. Minimum inhibitory concentration (MIC) of garlic ethanolic extract against food-associated bacteria on Mueller Hinton agar medium using macrodilution agar method.

Food associated bacteria	Concentration of clove ethanolic extract (mg/ml)								
	1.25	2.5	5.0	10.0	20.0	40.0	80.0	160.0	MIC
Bacillus subtilis	+	+	+	+	-	-	-	-	10.0
B. megaterium	+	+	+	+	-	-	-	-	20.0
B. sphaericus	+	+	+	-	-	-	-	-	10.0
B. polymyxa	+	+	+	-	-	-	-	-	10.0
Staphylococcus aureus	+	+	+	-	-	-	-	-	10.0
Escherichia coli	+	+	+	+	-	-	-	-	20.0

+ Growth; - No growth

Table 5. Minimum inhibitory concentration (MIC) of clove ethanolic extract against food-associated fungi using modified microdilution tube method.

Food associated fungi	Concentration of clove ethanolic extract (mg/ml)								
	1.25	2.5	5.0	10.0	20.0	40.0	80.0	160.0	MIC
Aspergillus luchuensis	+	+	+	-	-	-	-	-	10.0
A. flavus	+	+	+	+	-	-	-	-	20.0
Penicillium oxalicum	+	+	+	+	-	-	-	-	20.0
Rhizopus stolonifer	+	+	+	-	-	-	-	-	10.0
Mucor sp.	+	+	+	-	-	-	-	-	10.0
Scopulariopsis sp.	+	+	+	+	-	-	-	-	20.0

+ Growth; - No growth

Table 6. Minimum inhibitory concentration (MIC) of garlic ethanolic extract against food-associated fungi using modified microdilution tube method.

Food associated fungi	Concentration of clove ethanolic extract (mg/ml)								
	1.25	2.5	5.0	10.0	20.0	40.0	80.0	160.0	MIC
Aspergillus luchuensis	+	+	+	+	-	-	-	-	20.0
A. flavus	+	+	+	+	-	-	-	-	20.0
Penicillium oxalicum	+	+	+	+	-	-	-	-	20.0
Rhizopus stolonifer	+	+	+	-	-	-	-	-	10.0
Mucor sp.	+	+	+	-	-	-	-	-	10.0
Scopulariopsis sp.	+	+	+	+	-	-	-	-	20.0

+ Growth; - No growth

Conclusions

It may be suggested from the findings that both the clove and garlic ethanolic extracts can be used as a potential source of natural antimicrobial compound which if applied to bakery products. Further research is needed 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 nutraceutical foods.

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