

Comparison Of Antimicrobial Activities Of Clove Oil & Its Extract On Some Food Borne Microbes

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Citation

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Abstract

A study was carried out in the comparative analysis of ethanolic extract (50%) of clove & clove oil as natural antimicrobial agents on some food spoilage bacteria. The antibacterial activity of clove oil & its extract (50% ethanol) was tested against ten bacteria (seven Gram positive & three Gram negative) & seven fungi by agar well diffusion assays. The clove oil was found to be better antagonistic agent as compared to its extract counterpart by inhibiting both bacteria & fungi. The oil was found to be very effective with a lowest minimum inhibitory concentration (MIC) of 2.5% (v/v) against *Staphylococcus epidermidis* & *Staphylococcus sp.* Amongst the fungi, *Aspergillus niger* was found to be highly sensitive to the oil. Sodium propionate (standard food preservative) was used as a positive control. Clove oil was found to be more effective as compared to both clove extract & sodium propionate. This study shows the potential of clove oil to be used as food bio-preservative.

INTRODUCTION

The increase of food borne infections has resulted from the consumption of food contaminated with bacteria and/or their toxins. This has initiated considerable research interest towards the discovery of potent antimicrobial agents. Today the issue of food preservation has become more complex, with increasing concern over the presence of chemical residue in foods and the demand for non-toxic natural preservatives is increasing everyday because of harmful effects of food preservatives. The recent negative consumer perception against artificial preservatives, however, has shifted the research effort towards the development of alternatives that consumers perceive as naturals (Shelef, 1984). Antimicrobial properties of herbs and spices have been recognized and used since ancient times for food preservation as well as in medicinal use (Zaika, 1988; Conner, 1993; Dorman, 2000). A renewed interest in 'natural preservation' appears to be stimulated by present food safety concerns, growing problems with microbial resistance and rise in the production of minimal processed food joined with 'green' image policies of food industries.

Cloves (*Syzygium aromaticum*, syn. *Eugenia aromaticum* or *Eugenia caryophyllata*) are the aromatic dried flower buds of a tree in the family Myrtaceae (Srivastava & Malhotra, 1991; Chaieb et al., 2007a). Cloves are used in Ayurveda, Chinese medicine and Western herbalism. Cloves are used as a

carminative, to increase hydrochloric acid in the stomach and to improve peristalsis (Phyllis & James, 2000). It is also used in dentistry where the essential oil of clove is used for dental emergencies (Cai & Wu, 1996; Prashar et al., 2006). In addition, the cloves are anti-mutagenic (Miyazawa & Hisama, 2003), anti-inflammatory (Kim et al., 1998), antioxidant (Chaieb et al., 2007b), anti-ulcerogenic (Bae et al., 1998; Li et al., 2005), anti-thrombotic (Srivastava & Malhotra, 1991) and anti-parasitic (Yang et al., 2003).

The chief significance of the above study was to carry out the comparative analysis of the antimicrobial activities of clove oil & extract as an alternative to chemical preservatives for improving the shelf life of food products.

MATERIALS AND METHODS

Materials: All chemicals used were of analytical-reagent grade and obtained from E. Merck (Mumbai, India). Readymade clove oil & clove buds (*Syzygium aromaticum*) were purchased from local market of Meerut (Uttar Pradesh, India). Dr. C.M Govil, Professor, Botany Department, C.C.S University, Meerut, India confirmed the species.

Bacterial and fungal test isolates: Ten bacterial isolates (seven Gram-positive and three Gram-negative), mostly food-borne pathogens, were selected for this study. The Gram-positive bacteria comprised *Bacillus cereus*, *Bacillus subtilis*, *Bacillus sp.*, *Staphylococcus aureus*, *Staphylococcus*

epidermidis, *Listeria monocytogenes* and *Micrococcus luteus*, while the Gram-negative bacteria comprised *Escherichia coli*, *Klebsiella sp.* and *Pseudomonas aeruginosa*. The fungal isolates used in this study were *Alternaria sp.*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus sp.*, *Penicillium sp.*, *Rhizopus sp.* and *Rhizomucor sp.* The bacterial and fungal cultures were obtained from the Department of Microbiology, C.C.S University. The bacteria and fungi were cultured on nutrient agar medium and Sabouraud's dextrose agar (SDA) medium (Hi-Media, Mumbai, India), respectively. The agar plates were incubated at 37°C for 24 h (bacteria) and at 28°C for 3days (fungi). The stock on nutrient agar medium (Hi Media, Mumbai, India) was incubated for 24h at 37°C following refrigeration storage at 4°C until required for sensitivity testing. The viability tests for each isolate were carried out by resuscitating the organism in nutrient agar medium.

Extraction: The buds of clove (*Syzygium aromaticum*) was dried and grounded in a milling machine (Inalsa Mixer Grinder) to obtain a fine dry powder. The powder was weighed, using a single pan electronic weighing balance (Ohaus) and the clove extract was obtained by maceration process. The clove powder was soaked in 50% ethanol (1g of powder per 5mL of solvent) in a 250mL Erlenmeyer flask for 48h at room temperature with intermittent shaking. The flasks were closed with cotton plug and aluminium foil. The mixture was centrifuged at 3,500xg for 20min and finally filtered through Whatmann filter paper No.1 (Azoro, 2000). The pellet was discarded and the supernatant was collected and concentrated under reduced pressure in a rotary vacuum evaporator (Buchi) until a semisolid substance was obtained. This was dried inside the crucible under a controlled temperature (45°C) to obtain solid powder (Jonathan and Fasidi, 2003). The process of extraction was repeated until the weight of 500mg was obtained.

The powder was weighed and reconstituted in dimethyl sulfoxide (DMSO). These were stored in the refrigerator at 4°C for testing antimicrobial sensitivity. Once the extracts were dissolved in pure DMSO, these are also sterilized, and thus, a very costly and time-consuming step of membrane filtration sterilization was omitted (Zgoda and Porter, 2001). The extract was also exposed to UV rays for 24h and checked for sterility by streaking on NAM.

Antibacterial assay: The antimicrobial activities of clove extract and oil were determined by agar well diffusion method (Okeke et al., 2001). Briefly, pure isolate of each

bacterium was first sub-cultured in nutrient broth at 37°C for 24h. One hundred microlitres (100µL) of the standardized inoculum (106CFU/mL; 0.5 Mac-Farland) of each test bacterium was spread with the help of sterile spreader on to a sterile Muller-Hinton Agar plate (Hi Media) so as to achieve a confluent growth. The plates were allowed to dry and a sterile cork borer of diameter 6.0mm was used to bore wells in the agar plates. Subsequently, a 50µL volume of the extract and the oil was introduced in triplicate wells into Muller-Hinton Agar plate. Sterile DMSO served as negative control. A positive control in the form of sodium propionate (standard food preservative) was also included in the study. The plates were allowed to stand for atleast 1h for diffusion to take place and then incubated at 37°C for 24h. The zone of inhibition was recorded to the nearest size in mm (Norrel and Messely, 1997).

Figure 1

Table1: Zone of inhibition (mm) of clove extract () and clove oil on Mueller- Hinton agar medium

S.No.	Test bacterial species	Clove extract	Clove oil	Positive control	Negative Control
1.	<i>Bacillus cereus</i>	15.0	24.0	20.0	0.0
2.	<i>B. subtilis</i>	16.0	18.0	14.0	0.0
3.	<i>Bacillus sp.</i>	17.0	24.0	14.0	0.0
4.	<i>Staphylococcus aureus</i>	26.0	18.0	17.0	0.0
5.	<i>Staphylococcus epidermidis</i>	19.0	20.0	14.0	0.0
6.	<i>Listeria monocytogenes</i>	12.0	21.0	12.0	0.0
7.	<i>Micrococcus luteus</i>	14.0	16.0	14.0	0.0
8.	<i>Escherichia coli</i>	14.0	17.0	12.0	0.0
9.	<i>Klebsiella sp.</i>	13.0	15.0	12.0	0.0
10.	<i>Pseudomonas aeruginosa</i>	0.0	0.0	11.0	0.0

Incubation temperature: 37°C; Incubation period: 24h

Negative control- Dimethyl sulfoxide; Positive control- Sodium propionate

Volume of extract/ oil in each well = 50µL

Each value is the average of three independent replicates

Antifungal assay: For determining the antifungal activity of the clove extract and oil, the fungal isolates were sub-cultured on SDA at 28°C for 3 – 4 days. Sterilized Sabouraud's Dextrose Agar plates were taken and a sterile cork borer (6-mm diameter) was used to bore wells in the agar. A 50µL volume of the extract and oil was introduced

into each of the peripheral wells, while a fungal disc was inoculated into the central well. A negative control (sterilized DMSO) was also included in one of the peripheral wells to compare the activity. The plates were then incubated at 28°C. The evaluations were carried out by means of daily measurement of the colony diameter, starting at 24 h after the experiment began and finishing when two-thirds of the plate surface of the control treatment was covered by the fungus (Fiori et al., 2000). The appearance of zones of inhibition was regarded as positive for the presence of antimicrobial action in the test substance.

The results were expressed in terms of the diameter of the inhibition zone: < 9 mm, inactive; 9 – 12 mm, partially active; 13 – 18 mm, active; >18 mm, very active (Junior and Zani, 2000).

Figure 2

Table 2: Zone of inhibition (mm) of clove extract () and clove oil against common food spoilage fungi on SDA medium

S.No.	Test fungal species	Clove extract	Clove oil	Positive control	Negative Control
1.	<i>Alternaria sp.</i>	30.0	28.0	27.0	0.0
2.	<i>Aspergillus fumigatus</i>	20.0	30.0	17.0	0.0
3.	<i>Aspergillus niger</i>	27.0	42.0	16.0	0.0
4.	<i>Aspergillus sp.</i>	21.0	36.0	15.0	0.0
5.	<i>Penicillium sp.</i>	22.0	40.0	30.0	0.0
6.	<i>Rhizopus sp.</i>	29.0	40.0	18.0	0.0
7.	<i>Rhizomucor sp.</i>	26.0	35.0	20.0	0.0

Negative control- Dimethyl sulfoxide; Positive control- Sodium propionate

Volume of extract/ oil in each well = 50µL

Each value is the average of three independent replicates

Determination of minimum inhibitory concentration (MIC) of the extract and oil: The MIC was defined as the lowest concentration that completely inhibited the growth for 24 h (Thongson et al., 2004). The MIC for the crude extract was determined by the agar well diffusion method. A two-fold serial dilution of the clove oil, extract and sodium propionate was prepared by first reconstituting it in DMSO. It was then diluted in sterile DMSO to achieve a decreasing concentration range of 500 to 62.5 mg/ml. For clove oil, a decreasing concentration range of 10 to 0.625% (v/v) was prepared. A 50 µL volume of each dilution was added aseptically into the wells in Mueller Hinton agar plates that had been inoculated with standardized inoculums (10⁶ CFU/mL) of the test bacteria. The agar plates were incubated at 37°C for 24 h. Sodium propionate only served as positive

control. All experiments were performed in triplicate. The same procedure was used for fungi, except that SDA plates were used and the plates were incubated at 28°C. The lowest concentration of clove extract and oil showing a clear zone of inhibition was considered as the MIC.

Figure 3

Table 3: The MIC values of clove extract (mg/mL) and clove oil (%v/v) on Mueller- Hinton agar medium

S.No.	Test bacterial species	Clove extract (mg/mL)	Clove oil (%v/v)	Sodium propionate (mg/mL)
1.	<i>Bacillus cereus</i>	100	5	125
2.	<i>B. subtilis</i>	200	5	250
3.	<i>Bacillus sp.</i>	50	5	62.5
4.	<i>Staphylococcus aureus</i>	12.5	5	125
5.	<i>Staphylococcus epidermidis</i>	100	2.5	500
6.	<i>Listeria monocytogenes</i>	100	5	500
7.	<i>Micrococcus luteus</i>	200	10	500
8.	<i>Escherichia coli</i>	100	5	500
9.	<i>Klebsiella sp.</i>	100	5	500
10.	<i>Pseudomonas aeruginosa</i>	-	-	1000

Each value is the average of three independent replicates

Figure 4

Table 4: The MIC values of clove extract (mg/mL) and clove oil (%v/v) against test fungi on SDA medium

S.No.	Test fungal species	Clove extract (mg/mL)	Clove oil (%v/v)	Sodium propionate (mg/mL)
1.	<i>Alternaria sp.</i>	5000	5	1000
2.	<i>Aspergillus fumigatus</i>	7000	10	4000
3.	<i>Aspergillus niger</i>	6000	2.5	4000
4.	<i>Aspergillus sp.</i>	8000	5	2000
5.	<i>Penicillium sp.</i>	3000	2.5	500
6.	<i>Rhizopus sp.</i>	3000	2.5	1000
7.	<i>Rhizomucor sp.</i>	7000	5	1000

Each value is the average of three independent replicates

RESULTS

INHIBITORY ACTIVITY AGAINST BACTERIA

Clove extract was found to be effective against almost all of the food borne microbes. *Staphylococcus aureus* was found to be the most sensitive to clove extract with an inhibition zone diameter (IZD) of 26mm, followed by *Staphylococcus epidermidis* (19mm). *Bacillus cereus*, *B. subtilis* & *Bacillus sp.* were also found to be sensitive against clove extract with an IZD of 15, 16 and 17mm respectively. However, *Pseudomonas aeruginosa* was found to be resistant, as shown in Table 1. Similarly, clove oil also inhibited the growth of almost all test bacteria except *P. aeruginosa*, it produced the

widest zone of inhibition against *Bacillus cereus* & *Bacillus* sp. (24 mm). However, the MIC values of clove oil were much better than the clove extract. The values ranged from 2.5 to 10%v/v for clove oil and between 12.5- 100mg/mL for clove extract. The lowest MIC (2.5% v/v) of clove oil was against *Staphylococcus epidermidis* and for clove extract the lowest MIC was 12.5mg/mL against *Staphylococcus aureus* (Table 3). *Bacillus* sp. was found to be very sensitive to sodium propionate with MIC of 62.5mg/mL.

INHIBITORY ACTIVITY AGAINST FUNGI

With regard to antifungal activity, the clove extract resulted in the widest IZD against *Alternaria* sp. (30 mm) and was active against all the test fungal species (Table 2). Similarly, clove oil inhibited the growth of all test fungi. The widest IZD was produced against *Aspergillus niger* (42 mm) followed by *Rhizopus* sp. and *Penicillium* sp. with an IZD of 40 mm each (Table 2). Sodium propionate demonstrated a moderate spectrum of activity against all test fungi. *Penicillium* sp. was found to be highly sensitive to sodium propionate with an IZD of 32 mm (Table 2).

For the fungi, the MIC values of clove extract, oil & sodium propionate exhibited a broad range. *Rhizopus* sp. and *Penicillium* sp. was found to be highly sensitive to the clove extract by showing the lowest MIC of 3000mg/mL. For sodium propionate, the MIC values ranged between 500 to 4000mg/mL. Thus sodium propionate was found to be more effective as an antifungal agent compared to clove extract. In contrast, clove oil was active against all test fungi and the MIC values ranged between 2.5 to 10% (v/v), with the lowest MIC of 2.5% (v/v) each against *A. niger*, *Rhizopus* sp. and *Penicillium* sp. as shown in Table 4.

DISCUSSION

The inhibitory activity of clove is due to the presence of several constituents, mainly eugenol, eugenyl acetate, beta-caryophyllene, 2-heptanone (Chaieb et al., 2007b), acetyl-eugenol, alpha-humulene, methyl salicylate, iso-eugenol, methyl-eugenol (Yang et al., 2003), phenyl propanoides, dehydrodieugenol, trans-confireryl aldehyde, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid and oleanolic acid (Cai & Wu, 1996). The main constituents of essential oil are phenyl-propanoides such as carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb et al., 2007a). Several studies have demonstrated potent antifungal (Arina & Iqbal, 2002; Giordani et al., 2004; Pawar & Thaker, 2006; Park et al., 2007), antiviral (Chaieb et al., 2007a) and antibacterial effects of clove (Cai & Wu, 1996; Bae et al.,

1998; Lopez et al., 2005; Li et al., 2005; Betoni et al., 2006; Fu et al., 2007).

The modes of action by which microorganisms are inhibited by essential oil and their chemical compounds seem to involve different mechanisms. It has been hypothesized that the inhibition involves phenolic compounds, because these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing increased permeability, unavailability of vital intracellular constituents (Juven et al., 1994) and/or impairment of bacterial enzymes systems (Frag et al., 1989).

The components with phenolic structure such as eugenol are highly active against the test microorganisms. Clove oil has 79.2% eugenol (Ranasinghe et al., 2002). Members of this class are known to be either bactericidal or bacteriostatic agents, depending upon the concentration used (Pelczar et al., 1988, Dorman et al., 2000). These compounds were strongly active despite their relatively low capacity to dissolve in water, which is in agreement with published data (Sivropoulou et al., 1996; Hili et al., 1997; Lis-Balchin and Deans 1997).

The high activity of the phenolic compounds/ fractions may be further explained in terms of the alkyl substitution into the phenol nucleus, which is known to enhance the antimicrobial activity of phenols (Pelczar et al., 1988, Dorman et al., 2000, Campo et al., 2003).

CONCLUSION

In summary, this study confirms that clove oil possess much better in vitro antibacterial and antifungal activity than its extract and sodium propionate. Hence, it represents an alternative source of natural antimicrobial substances for use in food systems to prevent the growth of food-borne bacteria and extend the shelf-life of the processed food. However, further analysis of clove could be done to isolate the antimicrobial agents present in the spice and to determine their minimal inhibitory concentrations so that they can be used as bio-preservatives in various food stuffs.

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