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Bioactive Compounds and its Autochthonous Microbial Activities of Extract and Clove Oil (*Syzygium aromaticum* L.) on Some Food Borne Pathogens

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ABSTRACT

In the present study, we evaluated antimicrobial activity of clove oil against a range of food borne pathogens like bacteria and fungal autochthonous microorganisms and study the bioactive compounds present in the clove oil (dry) a nd its bioactivity. The *Syzygium aromaticum* Clove oil (extracted) and acetone based extracted clove oil analyzed by GC-MS. It contains a three higher peak as well as three lowest peak important phytochemical compounds such as Eugenol, a-cubebene, Iso-eugenitol and a-copaene, β-Caryophyllene oxide and β-Bipinene, respectively. The GC-MS analysis showed the presence of various secondary bioactive compounds and its antimicrobial activity of dried clove oil and crude extract against pathogenic bacteria like Gram positive (seven) and three Gram negative (*Escherichia coli*, *Salmonella* sp. and *Klepsiella pneumoneae*). This results showed that the relative analysis of acetone extract (50%) of crude clove extract and clove oil as a natural autochthonous antimicrobial agent on some food spoilage Gram negative as well as Gram positive bacteria. The oil was found to be very effective with a lowest minimum inhibitory concentration (MIC) of 2.5% (v/v) against *Staphylococcus epidermidis* and *Staphyl* sp. amongst the fungi, *Aspegillus niger* and *Rhizopus* sp., was found to be highly sensitive to the oil. Sorbic acid (std food preservative) was used as a positive control. Clove oil was found to be more effective when compared to both clove extract and Sorbic acid. This study shows the potential of clove oil to be used as food bio-preservative.

Key words: *Syzygium aromaticum*, autochthonous microbes, antifungal activity

INTRODUCTION

Clove oil has a long history of use as natural microbial agents. It has recently been used in a number of pharmaceutical food and cosmetic products because these oils effectively inhibit the growth of a wide range of microorganisms. Thus, many researchers have recently attempted to identify the autochthonous antimicrobial properties of essential oil. Clove oils have been widely investigated due to their popularity availability and high essential oil content. 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. Importantly, Lee and Shibamoto (2001) reported that clove oil might also be used as an anti-carcinogenic agent due to its antioxidant properties. Their results also indicate that the clove oil might be of use as a potential chemopreventative agent. 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 and 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 and James, 2000). It is also used in dentistry where the essential oil of clove is used for dental emergencies (Mytle *et al*., 2006; Prashar *et al*., 2006). In addition, the cloves are anti-mutagenic (Miyazawa and Hisama, 2003), antioxidant (Chaieb *et al*., 2007b), anti-ulcerogenic (Li *et al*., 2005) 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 and 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) clove oil (by steam distillation) and dried clove buds (*Syzygium aromaticum*) were purchased in mid April 2009 from local market of Kollemcode (Kanyakumari, India).

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*, *S. feacalis* and *Micrococcus luteus*, while the Gram-negative bacteria comprised *Escherichia coli*, *Klebsiella* sp. and *Pseudomonas aeruginosa* and *Salmonella* sp. The fungal isolates used in this study were *Candida albicans*, *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, Vivek, laboratory, Nagercoil. 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 3 days (fungi). The stock on nutrient agar medium (Hi Media, Mumbai, India) was incubated for 24 h 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 dried buds of clove (Peduncle) (April) *A. Vasica* 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 (Mumbai) and the clove extract was obtained by maceration process with acetone solvent. The clove powder was soaked in 50% ethanol (1 g of powder per 5 mL of solvent) in a 250 mL Erlenmeyer flask for 48 h at room temperature with intermittent shaking. The flasks were closed with cotton plug and aluminium foil. The mixture was centrifuged at 3,500 mg for 20 min 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 500 mg 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 24 h 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 24 h. One hundred microlitres (100 μL) of the standardized inoculum (106 cfu mL^{-1}; 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 6.0 mm was used to bore wells in the agar plates. Subsequently, a 50 μL 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 at least 1h for diffusion to take place and then incubated at 37°C for 24 h.

- Negative control-Dimethyl sulfoxide; Positive control- Sodium propionate μL Volume of extract/ oil in each well = 50. 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 50 μL 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 Zanil, 2000).

Statistical analysis: Paired sample t-test was used to determined the significance between experimental autochthonous bacteria against the clove oil and clove crude extract.

Identification of active antifungal fractions by GC/MS analysis: The essential oil fractions that showed antifungal activity were analyzed by gas chromatography/mass spectrometry (GC/MS) in order to examine their chemical structure. The GC (HP 6890) conditions for clove oil were as

follows : HP-5 MS column (30 m×0.25 mm×0.25) the injector and detector temperatures were maintained at 250 and 280°C, respectively; helium was used as the carrier gas at flow rate of 1 mL min^{-1} the oven temperature was initially maintained at 60°C for 5 min and then raised to 280°C at a rate of 5°C min^{-1} and for 5 min. Mass spectrometry (HP 5973) was used in ei mode ionization voltage, 70 eV; emission current, 40 μA; scan rate, 1 scans; scan range, 35-700 m z^{-1} and ion source temperature 200°C. The chemical structure of each constituent was identified by comparing the mass data for their peaks with standard library data.

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 26 mm, followed by *Staphylococcus epidermidis* (19 mm). *Bacillus cereus*, *B. subtilis* and *Bacillus* sp., were also found to be sensitive against clove extract with an IZD of 15, 16 and 17 mm, respectively. However, *P. 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* and *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 12.5-100 mg mL^{-1} for clove extract. The lowest MIC (5.5% v/v) of clove oil was against *Staphylococcus epidermidis* and for clove extract the lowest MIC was 125 mg mL^{-1} against *Staphylococcus aureus* (Table 3). *Bacillus* sp. was found to be very sensitive to sodium propionate with MIC of 62.5 mg mL^{-1}. From the Table 1 revealed the *Staphylococcus*, *Bacillus* and *Micrococcus* species expressed the significant activities react with the clove oil and its bioactive compounds. Remaining species of the microbes showed strongly insignificant. Similarly among the 6 fungal organisms statistically significant has been clearly showed on the three species like *Aspergillus*, *Penicillium* sp. and finally *Rhizomucor* sp., Meanwhile, when fungal organisms treated with clove oil that indicated highly significant on only one species of Aspergillus than the other fungi (Table 2).

Inhibitory activity against fungi: *Paeciliomyces* sp. (27 mm) then 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 *Apergillus niger* (42 mm) followed by *Rhizopus* sp. and *Penicillium* sp.,

Table 1: Zone of inhibition (mm) of clove extract (*Syzygium aromaticum*) and clove oil on Mueller-Hinton agar medium

Experimental bacterial species	Dried clove crude extract (mg mL^{-1})	Oil extract (%v/v)	Positive control	Negative control
Bacillus subtilis	13.0	23.0	20.0*	0.0
B. cereus	16.0*	15.0^{is}	15.0	0.0
Staphy. faecalis	19.0	16.0*	18.0	0.0
S. aureus	26.0*	19.0**	15.0*	0.0
S. epidermidis	19.0*	21.0**	17.0	0.0
Micrococcus luteus	17.0*	15.0^{ns}	18.0**	0.0
K. pneumoniae	18.0*	17.0	11.0	0.0
E. coli	13.0*	18.0**	16.0	0.0
Pseudomonas aeruginosa	0.0	0.0	12.0	0.0
Salmonell sp.	15.0*	14.0**	13.0	0.0

*Indicates 5% significant level by paired sample t-test. **Indicates highly significant p≤0.001%. ^{is}Insignificant shows between clove oil and extract with test species of bacterial species

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

Experimental fungal species	Crude extract (mg mL^{-1})	Oil extract (%v/v)	Positive control	Negative control
Paeciliomyces	27.0^{is}	28.0^{is}	25.0*	0.0
Aspergillus flavus	20.0*	31.0**	18.0^{is}	0.0
Aspergillus niger	27.0*	41.0^{is}	16.0*	0.0
Penicillium sp.	21.0*	36.0^{is}	15.0**	0.0
Rhizopus sp.	29.0^{is}	40.0^{is}	18.0	0.0
Rhizomucor sp.	26.0*	35.0^{is}	20.0*	0.0

*Indicates significant level p≤0.05% by paired sample t-test. **Indicates highly significance, ^{is}- Indicates insignificance, Negative control- Dimethyl sulfoxide; Positive control- Sodium propionate, µL volume of extract/ oil in each well = 50

Table 3: The MIC values of clove extract (mg mL^{-1}) and clove oil (%v/v) on Mueller-Hinton agar medium

Test bacterial species	Crude extract (mg mL^{-1})	Oil extract (%v/v)	Sorbic acid (mg mL^{-1})
Bacillus subtilis	100*	7^{is}	125
B. cereus	200*	6^{is}	250
Staphy. faecalis	75*	9*	75.68
S. aureus	15.50**	8*	125
S. epidermidis	90^{is}	5.5**	250
Micrococcus luteus	150^{is}	7.64**	200
K.pneumoniae	100**	5.6^{is}	150
E.coli	100**	10^{is}	165
Pseudomonas aeruginosa	-	-	700
Salmonell sp.	150	5^{is}	400

Each value is the average of three independent replicates. *Indicates significant level p≤0.05% by paired sample t-test, **Indicates highly significance, ^{is}Indicates insignificance

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 Sorbic acid with an IZD of 32 mm (Table 2). From the statistical analysis clearly depicted when the 6 fungal organisms treated with crude extract, among them four species such as *A. niger*, *A. flavus*, *Penicillium* and *Rhizopus* species were shown statistically significant response on the clove crude extract remaining two species were shows insignificant response on the same extract. But in case of clove oil extract highly significant activity expressed with one fungal organism named as *A. flavus* than other five fungi.

For the fungi, the MIC values of clove extract, oil and Sorbic acid 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 300 mg mL^{-1}. Apart from this result Sorbic acid was the important food preservative agent, the MIC values ranged between 300 to 500 mg mL^{-1}. Thus, the Sorbic acid 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 3.5 to 14.7% (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.

GC-MS analysis of dried cloves: From the Fig. 1 indicated GC-MS analysis graph that shows almost three highest peak such a peak values were denoted bioactive compounds specific named as Eugenone, Iso-eugenitol and a-Cubebene showed highest abundance as well as retention

Table 4: The MIC values of clove extract (mg mL^{-1}) and clove oil (%v/v) against test fungi on SDA medium

Experimental fungal species	Clove acetone extract (mg mL^{-1})	Clove oil (%v/v)	Sorbic acid (mg mL^{-1})
Paeciliomyces	500*	14.7*	100
Aspergillus flavus	600**	8*	400
Aspergillus niger	600*	3.5^{is}	400
Penicillium sp.	500*	3*	300
Rhizopus sp.	400^{is}	3*	700
Rhizomucor sp.	700**	5**	700

Each value is the average of three independent replicates. *Indicates significant level $p \leq 0.05\%$ by paired sample t-test. **Indicates highly significant $p \leq 0.001\%$, ^{is}Insignificant shows between clove oil and extract with test species of fungal organisms

Table 5: GC-MS analysed compounds from the dried clove oil

No. of peak	Name of the analyte for leaves	Retention time (min)	Abundance (%)
1	Eugenone	1.87	35
2	a-Cubebene	1.24	25
3	a-Copaene	1.02	22
4	β-Caryophyllene oxide	1.35	12
5	Iso-eugenitol	1.54	36
6	β- Bipinene	1.85	Trace

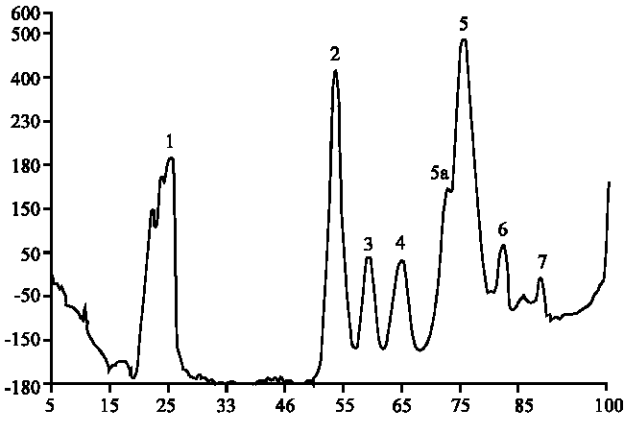

Fig. 1: Chromatograms for bio-active compounds present in the acetone extract of dried clove oil (*Syzygium aromaticum* L.)

time then the lowest peak exhibited the phytochemical compounds such as a-Copaene, **β**-Caryophyllene oxide. Though, the initially eugenol is an important phytochemicals and its retention time was 1.87 also it's having the abundance amount is 35 this was just lower than the Iso-euginitol abundance 36 (Table 5).

DISCUSSION

Several studies have also shown that the eugenol possesses antioxidant properties (Lee and Shibamoto, 2001) and can be used to alleviate fever (Feng and Lipton, 1987). Three peaks appeared constituently on the chromatogram of the antifungal fractions of clove oil contains three compounds (Table 5). The three major constituents were identified were identified as Eugenone, Isougenitol and a-Cubebene respectively by GC/MS analysis. Previously this kind of similar results has been observed by Park *et al.* (2007). 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). These results are not consistent with results presented by Raotonirainy and Lavendrine, (2005) who reported that the clove oil and crude extract performed better than euginol against *Aspergillus* sp., *Cladosporum herbarum* and *Penicillium frequentants*. The inconsistency in these results may be due to different levels of species sensitivity as well as different strengths of the clove oil and the commercial eugenol. The main constituents of essential oil are phenyl-propanoides such as carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb *et al.*, 2007a). This kind of consistent research findings have already been demonstrated by following authors with different types plant based oil (Arina and Iqbal, 2002; Giordani *et al.*, 2004; Pawar and Thaker, 2006; Park *et al.*, 2007), antiviral (Chaieb *et al.*, 2007a) and the same antibacterial effects of clove (Lopez *et al.*, 2005; Li *et al.*, 2005; Betoni *et al.*, 2006; Fu *et al.*, 2007) bioactive compounds and its potent antifungal activities.

The mode of action by which favorable as well as unfavorable microorganisms are inhibited by essential oil and their chemical compounds seem to involve different mechanisms, already been this kind of research opinion established by Thongson *et al.* (2004). 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 (Arora and Kaur, 1999). 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 (Dorman and Deans, 2000). The higher 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 (Dorman and Deans, 2000; Campo *et al.*, 2002).

CONCLUSION

These results suggest that the clove oil and crude extract may be viable alternatives to conventional antifungal and antibacterial agents with relatively minimal side effects. This study confirms that clove oil possess much better *in vitro* antibacterial and antifungal activity than its extract and good food preservative agent of Sorbic acid propionate. Hence, it represents an alternative source of natural autochthonous antimicrobial substances for use in food systems to prevent the growth of food-borne bacteria and fungi 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. However, the mechanism of the autochthonous microbial activity of essential oils has yet to be fully understood and therefore further research is needed in order to determined.

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