

Antimicrobial Activity of *Syzygium aromaticum* and Its Bud Oil Against Dental Caries Causing Microorganisms

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Abstract

The antimicrobial activity of clove and clove bud oil were investigated by agar well diffusion method against five dental caries causing microorganisms namely *Streptococcus mutans*, *Staphylococcus aureus*, *Lactobacillus acidophilus* (bacteria), *Candida albicans* and *Saccharomyces cerevisiae* (yeast). The results indicated that clove and clove oil have a potent antimicrobial activity against the tested dental caries causing microorganisms. The highest antimicrobial activity of clove was found against *Saccharomyces cerevisiae* (25.32mm) in methanolic extract and an MIC of 50mg/ml and that of clove oil was found against *Streptococcus mutans* (34.32mm) with a MIC value of 3.125mg/ml. This study has shown the importance of clove and clove oil and indicated that clove and clove bud oil can be used as an antimicrobial agent to cure dental caries.

Key words: Clove, clove oil, antimicrobial activity, inhibition zone, dental caries.

Introduction

Dental caries is a chronic disease of multifactor etiology and pathogens. The early stage of dental caries is characterized by a destruction of superficial dental structures caused by acids which are by-products of carbohydrate metabolism by *Streptococcus mutans*, a cariogenic bacterium (Loesche, 1986). Dental caries are one of the public health concerns for several reasons. Teeth affected with dental caries are sources of infection, which can cause an inflammation of dental pulp, periodontium and gums. If left untreated, this disease gradually leads to teeth loss, which causes chewing difficulties and aesthetic problems (Allen, 2003). It remains one of the most widespread diseases of the mankind. In developing countries, dental caries is often at epidemic proportions, especially among the poor. Since the 19th century, when sucrose became a daily used sweetener by many people worldwide, the increasing

prevalence of dental caries had also been noticed (Hamada, 2002). Despite a low mortality rate associated with dental diseases they have a considerable effect on the self esteem, eating ability, nutrition and health both in childhood and older age. In the modern society the most important role of teeth is to enhance facial appearance. Teeth also play an important role in speech and communication. Dental decay also leads to tooth loss which reduces the ability to eat a varied diet (Moynihan *et al.*, 1994; Steele *et al.*, 1998). Tooth loss has also been associated with loss of enjoyment of food and confidence to socialize (Steele *et al.*, 1998). It is therefore clear that dental diseases have detrimental effect on quality of life both in childhood and older age (Moynihan and Petersen, 2004).

Cloves (*Syzygium aromaticum*) are dried aromatic unopened floral buds of an evergreen tree 10-20 m in height, belonging to the family Myrtaceae, indigenous to India, Indonesia, Zanzibar, Mauritius and Ceylon (Ambasta, 1986; Chaieb *et al.*, 2007a). They are esteemed as a flavouring agent and also used as a spice for scenting, chewing tobacco and an ingredient of betel chew. Cloves have many therapeutic uses: they control nausea and vomiting, cough, diarrhea, dyspepsia, flatulence, stomach distension and gastro intestinal spasm, relieve pain, cause uterine contractions and stimulate the nerves (Elujoba *et al.*, 2005; Gordon, 1980; Phyllis and James, 2000; Sulieman *et al.*, 2007; Tanko *et al.*, 2008). In addition, the cloves are highly antiseptic (Blumenthal, 1998) antimutagenic (Miyazawa and Hisama, 2003), anti-inflammatory (Kim *et al.*, 1998), antioxidant (Chaieb *et al.*, 2007b), antiulcerogenic (Bae *et al.*, 1998; Li *et al.*, 2005), antithrombotic (Srivastava and Malhotra, 1991), antifungal (Giordani *et al.*, 2004; Park *et al.*, 2007), antiviral (Saeed and Tariq, 2008) and antiparasitic (Yang *et al.*, 2003).

Clove oil (*Syzygium aromaticum*) is widely used as a perfume and food flavouring (Zheng *et al.*, 1992; Kalemba and Kunicka, 2003), as a medicine for the treatment of asthma, rheumatoid arthritis, acne, warts, scars and various allergic disorders (Kim *et al.*, 1998), as an analgesic, anti spasmotic, and as a general antiseptic in medical dental practices (Cai and Wu, 1996). Clove bud oil, has been used for a long time by dentists as a dressing in dentistry for minor wounds, as an analgesic in painful and infective diseases of the oral cavity and pharynx as well as general hygiene. 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 suggested that clove oil might be of use as a potential chemopreventative agent (Zheng *et al.*, 2005). Research has shown that clove oil is an effective mosquito repellent (Trongtokit *et al.*, 2005). However, clove oil is toxic to human cells (Prashar *et al.*, 2006). If ingested or injected in sufficient quantity, it has been shown to cause life threatening complications, including Acute Respiratory Distress Syndrome, Fulminant Hepatic Failure and Central Nervous System

disorder. The lethal oral dose is 3.752 g/Kg body weight (Kirsch, 1990; Lane et al., 1991; Hartnoll et al., 1993).

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment. Over three-quarters of the world population relies mainly on plants and plant extracts for health care. The demand of plant based therapeutics is increasing in both developed and developing countries due to the growing recognition that they are natural products, being non narcotic, having no side effects, easily available at affordable prices and sometimes the only source of healthcare available to the poor. The objective of this study was to validate the observations made by earlier workers and to assess 1) the *in vitro* antibacterial and antifungal properties of different extracts of dried clove buds and clove oil against common dental caries causing microorganisms, 2) determination of minimum inhibitory concentration (MIC) of each extract and clove oil against each pathogen with a view of finding the minimum concentration of clove and its oil that can be assigned as a novel remedy for dental caries.

Materials and Methods

Clove buds and clove oil were collected from the local market of Delhi, India. Dr. B.D.Vashishta (Botany Department) Kurukshetra University, Kurukshetra confirmed the identification of cloves. Cloves are reddish- brown in color and have a strong aroma. Clove bud oil is a clear colorless to yellow mobile liquid, becoming browner with age, with strong characteristic sweet and spicy clove odour and a warm, almost burning spicy flavor.

Extraction

The samples were carefully washed under running tap water followed by sterile distilled water. These were air dried at room temperature (30°C) for two days and pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight bottles. Four different solvents namely ethanol, methanol, acetone and aqueous (hot and cold) were used for extraction. A 10g amount of pulverized buds was separately soaked in 100ml of acetone, ethanol, methanol, and cold sterile distilled water for 24h. Also the same amount (i.e. 10g) of pulverized buds were immersed in 100ml of hot sterile distilled water (100°C) and allowed to stand for 30min on a waterbath with occasional shaking and kept undisturbed for 24h. Each preparation was filtered through a sterilized Whatman No.1 filter paper and the filtered extract was concentrated under vacuum below 40°C using Heidolph, VE-11 rotaevaporator (Bag et al., 2009; Ogundiya et al., 2006). The dried extract thus obtained was exposed to UV rays for 24h and checked for sterility on nutrient agar plates and stored in labeled sterile bottles in a freezer at 4°C until further use (Nkere and Iroegbu, 2005).

Test Microorganisms

Three dental caries causing bacteria *Streptococcus mutans* (MTCC*497), *Staphylococcus aureus* (MTCC 740), *Lactobacillus acidophilus* (MTCC *447) and two yeasts *Candida albicans* (MTCC 227) and *Saccharomyces cerevisiae* (MTCC 170) were procured from Microbial Type Culture Collection, IMTECH, Chandigarh. The microorganisms were subcultured on the specific media recommended for different microorganisms such as Brain heart infusion agar (*S.mutans*), Nutrient agar (*S.aureus*), Lactobacillus MRS agar (*L.acidophilus*), Malt yeast agar (*C.albicans* and *S.cerevisiae*) and incubated aerobically at 37°C. The media were procured from Himedia Laboratory Pvt. Ltd., Bombay, India. Identification of all the strains was confirmed by standard biochemical and staining methods (Aneja, 2003; Benson, 2004, Cappuccino and Sherman, 1995).

Screening for Antimicrobial Activity

The acetone, methanol, ethanol, cold and hot water dried clove bud extracts were used for the screening. Antimicrobial activity of various extracts was determined by the agar well diffusion method (Okeke et al., 2001). In this method, pure isolate of each microbe was subcultured on the recommended specific media for each microorganism at 37°C for 24h. For testing clove oil, a final concentration of 0.5% (v/v) Tween-20 (Sigma) was incorporated into the agar after autoclaving to enhance oil solubility (Bansad, 2008). A plate of each microorganism was taken and a minimum of four colonies were touched with a sterile loop and transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted equal to that of 10⁶ cfu/ml (standardized by 0.5McFarland standard) and used as the inoculum for performing agar well diffusion assay. One hundred microlitres (100µl) of inoculum of each test organism was spread onto the specific media plates so as to achieve a confluent growth. The agar plates were allowed to dry and wells or cups of 8mm were made with a sterile borer in the inoculated agar plates and the lower portion of each well was sealed with a little specific molten agar medium. The extracts were reconstituted in 20% DMSO for the bioassay analysis (Rajasekaran et al., 2008). A 100µl volume of each extract was propelled directly into the wells (in triplicates) of the inoculated specific media agar plates for each test organism. The plates were allowed to stand for 10 minutes for diffusion of the extract to take place and incubated at 37°C for 24h (Khokra et al., 2008; Rios et al., 1980). Sterile DMSO served as the negative control and ciprofloxacin (for bacteria) and amphotericin-B (for fungi) served as the positive control. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone of inhibition was greater than 8mm (Hammer et al., 1999). The experiments were performed in triplicates and the mean

values of the diameter of inhibition zones with \pm standard deviation were calculated (Aneja and Joshi, 2009a, b).

Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of a compound/extract/drug that completely inhibits the growth of the microorganism in 24h (Thongson et al., 2004). The MIC for the acetic, methanolic and ethanolic extract was determined by following the modified agar well diffusion method (Okeke et al., 2001). A twofold serial dilution of each extract was prepared by first reconstituting the powder in 20% dimethylsulphoxide (DMSO) followed by dilution in sterile distilled water to achieve a decreasing concentration range of 50mg/ml to 0.39mg/ml. A 100 μ l volume of each dilution was introduced into wells (triplicate) in the specific media agar plates already seeded with 100 μ l of standardized inoculum (10^6 cfu/ml) of the test microbial strain. All test plates were incubated aerobically at 37°C for 24 hrs and observed for the inhibition zones. The lowest concentration of each extract showing a clear zone of inhibition, considered as the MIC, was recorded for each test organism (Nkere and Iroegbu, 2005; Aneja et al., 2009).

Results and Discussion

Antibacterial properties of clove have already been reported (Chopra *et al*, 1982, Ueda *et al*, 1982, Watanabe *et al.*, 1985, Briozzo *et al*, 1989 and Islam *et al.*, 1990; Hoque et al., 2008), but in the present study we tested the antibacterial as well as the antifungal activity of clove and its oil against dental caries causing microorganisms.

The results of antimicrobial activities of ethanol, methanol, acetone and aqueous (hot and cold) extracts of clove buds and clove oil as well as the positive control ciprofloxacin (for bacteria) and amphotericin-B (for fungi) are presented in Table 1 and the MIC of the five extracts as well as clove oil against the test pathogens are presented in Table 2 . The antimicrobial activity of clove bud extracts on the agar plates varied greatly in different solvents. Both the positive controls produced significantly sized inhibition zones against the test bacteria (ciprofloxacin) and yeasts (amphotericin-B). However, the negative control produced no observable inhibitory effect. Of the five extracts screened for antibacterial activity, all the five showed antibacterial activity against *S.mutans*. The hot aqueous extract was most effective against *S.mutans* showing the highest zone of inhibition (22.65mm) followed by the cold aqueous (20.32mm), acetic extract (14.65mm), ethanolic extract (13.95mm) and methanolic extract (11.64mm). Clove oil showed the highest zone of inhibition 34.32mm against *S.mutans* (Figure 1a), which was even much higher than the positive control i.e. ciprofloxacin (27.32mm). *S.mutans* was found to be

most sensitive pathogen which survived upto 1.56mg/ml in clove oil, thus having a MIC of 3.125mg/ml followed by the hot and cold aqueous extract (12.5mg/ml) and the acetonic, methanolic and ethanolic extracts (25mg/ml). *S.aureus* was found to be comparatively more resistant than *S.mutans* as only the methanolic (21.32mm) and ethanolic (19.32mm) clove bud extracts showed antibacterial activity against it and it survived upto 12.5mg/ml thus having a MIC of 25mg/ml. These results are in agreement with those of another study reporting that clove essential oil exhibited antibacterial activity against a large number of methicillin-resistant *S. epidermidis* and *S. aureus* (Enzo and Susan, 2002). *L.acidophilus* was found to be completely resistant to the five solvent extracts of clove buds while the clove oil produced quite big inhibition zone with a diameter of 29.97mm which was greater than the diameter of positive control (25.65mm). *L.acidophilus* survived upto 3.125mg/ml concentration of clove oil thus having a MIC of 6.25mg/ml. Out of the five extracts screened for antifungal activity, acetone, methanol and ethanol showed antifungal activity against *Candida albicans* while methanol and ethanol showed antifungal activity against *Saccharomyces cerevisiae*. However, water extracts, both hot and cold, showed no activity against the test strains. The inhibition zones produced by the three solvents against *C.albicans* ranged between 20mm and 24mm. The methanolic extract produced the largest zone of inhibition, among the various clove extracts tested, i.e. 25.32mm zone against *S.cerevisiae* (Figure 1b), while the ethanolic extract produced a 17.96mm zone. Clove oil showed excellent antifungal activity against *S.cerevisiae* with a mean diameter of inhibition zone being 28.97mm (much greater than the positive control i.e. amphotericin-B, which produced a zone of 11.94mm) but no activity against *C.albicans*. *S.cerevisiae* was found to be comparatively more resistant than *C.albicans* as it survived upto 25mg/ml, thus having a MIC of 50mg/ml in all the three extracts tested.

Knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis of complex chemical substances. In addition, the knowledge of the chemical constituents of plants would further be valuable in discovering the actual value of folkloric remedies (Mojab et al., 2003). Chemically constituents may be therapeutically active or inactive. The ones which are active are called active constituents and the inactive ones are called inert chemical constituents (Iyengar, 1995).

Several constituents of clove have been identified, mainly eugenol, eugenyl acetate, beta-caryophyllene, 2-heptanone (Chaieb et al, 2007a; Cowan, 1999), acetyeugenol, alpha-humulene, methyl salicylate, isoeugenol, methyleugenol (Yang et al., 2003), phenyl propanoides, dehydrodieugenol, trans-confireryl aldehyde, biflorin, kaempferol, rhamnocitrin, myricetin,

gallic acid, ellagic acid and oleanolic acid (Cai and Wu, 1996). The main constituents of essential oil are phenylpropanoides such as carvacrol, thymol, eugenol and cinnamaldehyde (Chaieb et al., 2007b). GC-MS analysis of the oil extract shows eugenol (88.58%), eugenyl acetate (5.62%), β -caryophyllene (1.39%), 2-heptanone (0.93%), ethyl hexanoate (0.66%), humulene (0.27%), α -humulene (0.19%), calacorene (0.11%) and calamenene (0.10%) as the major constituent. Eugenol and caryo-phyllene are known to possess antibacterial and antifungal properties. Hence the antibacterial and antifungal properties demonstrated by clove and clove oil can be attributed to the compounds reported (Ayoola et al., 2008; Prashar et al., 2006; Pawar and Thaker, 2006; Lee and Shibamoto, 2002). The potential for developing antimicrobial drugs from plants appears rewarding, as it will lead to the development of a phytomedicine that will act more effectively against microorganisms. Therefore such screening experiments form a primary platform for further phytochemical and pharmacological studies that may open the possibilities of finding new clinically effective antimicrobial compounds.

Conclusion

On comparison of the antimicrobial activities of all the five *S.aromaticum* bud extracts and clove oil tested against the bacterial and fungal strains, it was finally concluded that clove oil emerged as the potent agent exhibiting even much higher antibacterial and antifungal activity than the standard antibacterial and antifungal drugs ciprofloxacin and amphotericin-B respectively. The need of the hour is to perform more and more screening of the natural products or plant parts as such screening experiments form a primary platform for further phytochemical and pharmacological studies that may open the possibilities of finding new clinically effective antifungal and antibacterial compounds against the dental caries pathogens and the resistant bacterial and fungal pathogens.

Table 1: Antimicrobial activity of Clove and clove oil against the test microorganisms.

Clove extracts (mg/ml)	Diameter of growth of inhibition zones (mm)				
	<i>Streptococcus mutans</i>	<i>Staphylococcus aureus</i>	<i>Lactobacillus acidophilus</i>	<i>Candida albicans</i>	<i>Saccharomyces cerevisiae</i>
Acetone	14.65 ^a ±0.57 ^b	-	-	20±0	-
Methanol	18.96±1	21.32±0.57	-	24±0	25.32±0.57
Ethanol	13.95±1	19.32±0.57	-	21.32±0.57	17.96±1

Hot water	22.65±0.57	-	-	-	-
Cold water	20.32±0.57	-	-	-	-
Clove oil	34.32±0.57	-	29.97±1	-	28.97±1
Ciprofloxacin (5 µg/ml)	27.32±0.57	34.66±0.57	25.65±0.57	Nt	Nt
Amphotericin B (100 units/ ml)	Nt	Nt	Nt	13±0	11.94±1
DMSO	0	0	0	0	0

(-) = no activity, Nt = not tested

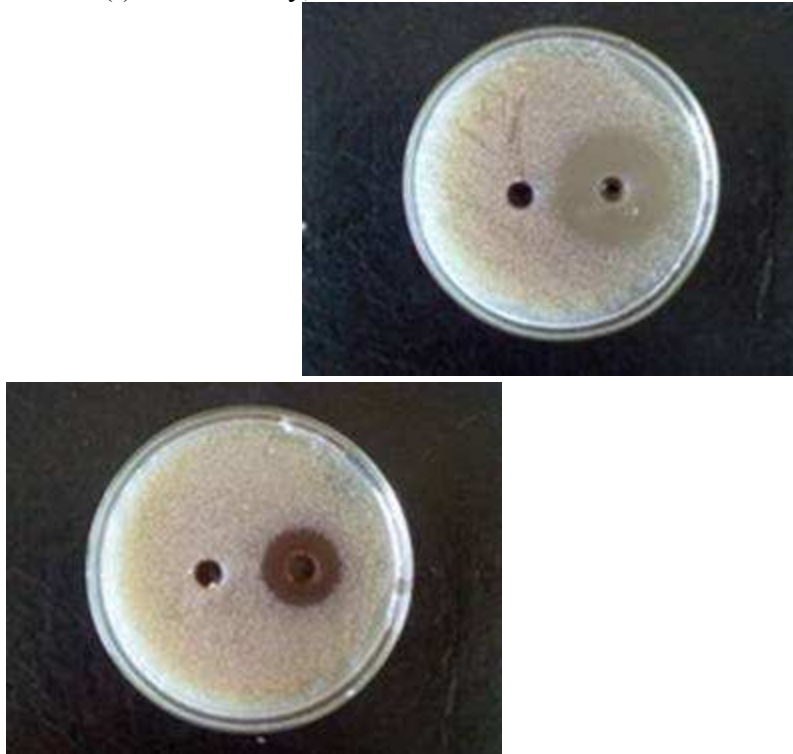
^a Values, including diameter of the well (8 mm), are means of three replicates

^b ± Standard deviation

Table 2: Minimum Inhibitory Concentration (MIC) of Clove and Clove oil.

Clove extracts	MIC (mg/ml)				
	<i>Streptococcus mutans</i>	<i>Staphylococcus aureus</i>	<i>Lactobacillus acidophilus</i>	<i>Candida albicans</i>	<i>Saccharomyces cerevisiae</i>
Acetone	25	Nt	Nt	50	-
Methanol	25	25	Nt	25	50
Ethanol	25	25	Nt	50	50
Hot water	12.5	Nt	Nt	-	-
Cold water	12.5	Nt	Nt	-	-
Clove oil	3.125	Nt	6.25	-	6.25

(-) = no activity, Nt = not tested



a.

b.

Figure 1: Zone of inhibition shown by (a) clove oil against *Streptococcus mutans* , (b) methanolic extract of clove against *Saccharomyces cerevisiae* and negative control (DMSO).

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