
Effect of Clove Oil on Adhesion of *Staphylococcus aureus* to Buccal Cavity Epithelial Cells *In vitro*

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Abstract:

Background: Adhesion is an important starting event in the pathogenesis of bacterial infection. The microorganisms must first adhere to host tissue in order to multiply and create a colony or colonies before symptoms allow the disease process to be detected. Clove oil has been reported to possess interesting antimicrobial effects on various microorganisms.

Objective: The objective of this study was to assess whether clove oil interferes with the adhesion of *S. aureus* to buccal epithelial cell.

Methods: Adults apparently healthy volunteers (forty) were included in this study. Adhesion assay has been carried out in this study by mixing *S. aureus* bacteria with the buccal epithelial cells and incubated with & without clove oil. Antibacterial activity of clove oil was determined by using disc diffusion assay as well as tube dilution assay.

Results: The percentage of epithelial cells with adhered bacteria decreased from 97.78% to 91.1% with a P value > 0.05, also the mean number of adhered bacteria per epithelial cells was decreased from 116.76 to 12.55 after oil treatment with a P value < 0.05. The antibacterial test revealed that the minimal inhibitory concentration for *S. aureus* was 1/256 (0.391(v/v)) of the original clove oil preparation

Conclusion: Clove oil which has antibacterial activity to *Staphylococcus aureus*, has an inhibitory effect on adhesion of *Staphylococcus aureus* to buccal epithelial cells

Key words: Clove oil, *Staphylococcus aureus*, adhesion, buccal epithelial cells. Control group were enrolled in the present

Introduction:

Bacterial adhesion to host cells is the initial and essential first step in all infectious processes ^[1].

Among gram – positive pathogens, surface protein represents the largest group of adhesions, although other factors such as polysaccharide or lipid may also display adhesive functions. Targets for these microbial adhesions are host molecules found on mucosal surface, skin and wound. Most Gram positive pathogens express multiple adhesions that may bind to either the same or distinct target molecules ^[2].

Staphylococcus aureus causes various superficial and systemic infections and is often implicated in oral mucositis, including angular cheilitis and denture – induced stomatitis ^[3].

Oral bacteria play an important role in the formation of dental caries and development periodontal disease.

These microorganisms colonize the tooth surface and initiate plaque formation. Dental plaque is one of the factors that can lead to the formation of dental caries ^[4].

Some of the more common bacterial species responsible for caries production include *Streptococcus mutans*, *Streptococcus sorbinus* and *Lactobacillus casei* which can perform glycolysis at a pH level as low as 3.0 Adhesion of bacteria to epithelial cells has different approaches compared to dental caries which results from adhesion of microorganisms to dental surfaces.

Staphylococcus aureus, a human pathogen produces a large number of proteins that specifically bind to molecules from plasma or from the human extracellular matrix (ECM). The interactions have been proposed to contribute to the colonization of host tissues. *S. aureus* has been shown to bind to fibronectin (Fn) ^[5], collagen, fibrinogen, vitronectin and elastin. Many bacterial proteins, that are associated with the cell wall exhibit a common amino acids sequence, an LPXTG motif, which anchors the molecule to the cell wall peptidoglycan ^[6,7]. Bacterial adhesion forces significantly decreased after coated with saliva, and increased with adhesion times ^[8].

Interaction of staphylococcal protein A and cytoskeletal actin filaments is involved in the *S. aureus* invasion of cultured KB cells, and this process may contribute, in part to the intracellular movement, cell-to-cell spread and dissemination of *S. aureus* within human oral epithelial cells in vivo^[9] In many parts of the world there is a rich tradition in the use of natural products for the treatment of many infectious diseases. Cloves are the dried unopened inflorescence of the clove tree *Syzygium aromaticum*, clove is a member of the Myrtaceae family^[10].

The essential oil extracted from the dried flower buds of cloves is used for acne, warts, scars and parasites^[11]. Research has shown that clove oil is an effective mosquito repellent^[12]. The clove oil is also used as a typical application to relieve pain and to promote healing and also used in the fragrance and flavoring industries^[11]. It's commonly used by some dentist to relieve tooth pain^[13]. Some workers confirmed the role of clove oil in antibacterial activity, but from the best of our knowledge, no previous work has been done on the role of this oil extracted from dried flower buds of clove plants on *S. aureus* adhesion to buccal cavity epithelial cell.

The aim of this study is to assess the role of the local pure clove oil extract in the process of *S. aureus* adhesion to buccal epithelial cell which represent the initial step in oral pathology and also to evaluate the antibacterial activity of this local oil extract which is commonly used by the dentists in Iraq.

Materials & Methods:

This study was conducted in Baghdad for the period from January to May 2010

Clove Oil Extract:

This pure product was prepared by the manufacturer without any additives as declared by the manufacturer. It were purchased from local herb shop in Baghdad (manufactured by Emad factory for oil extraction-Mosel) which is commonly used by the dentist. Clove oil was diluted in a half-fold dilution series^[14] with tryptic soy broth to achieve a decreasing concentration range of 50% to 0.049%(v/v)

used for MIC test in addition to prepare 1/3 dilution (0.333%(v/v)) for adherence assay

Bacterial strain:

The test microorganism used throughout the study was *Staphylococcus aureus*. These bacteria were isolated from buccal oral cavity.

The identity of the culture was confirmed by standard bacteriological methods^[15].

Preparation of bacterial suspension:

To prepare bacteria for adherence studies, suspension of organisms were prepared from overnight culture in tryptic soy broth at 37 °C under static conditions. The organisms were harvested, washed three times in PBS and re-suspended in 5ml of PBS to a concentration of 10⁹ bacteria per ml of PBS. Bacteria were counted by direct microscopic counts in an improved Neubauer Chamber instead of Petroff-Hausser chamber used by Dal, (2006)^[16].

Collection and preparation of buccal epithelial cells; human buccal epithelial cells were collected by gently scraping the oral mucosa of human volunteers with sterile swabs; scraped cells were suspended in 5ml of PBS. These cell suspensions were centrifuged (3000 rpm for 15min), washed three times with PBS and suspended to a concentration of 5x10⁶ epithelial cells per ml of PBS using an improved Neubauer Chamber.

In vitro adhesion assay:

The ability of bacteria to adhere to buccal epithelial cell in the presence or absence of 1/3 dilution of clove oil was investigated. This assay was done by mixing 0.5 ml epithelial suspension of 5x10⁶ cells per ml of PBS with organisms 0.5ml of bacterial suspension of 10⁹ bacteria per ml of PBS in polystyrene tubes. The tubes were incubated in water bath at 37°C for one hour with a gently shake each 20 minutes. The non adhered bacteria were separated from epithelial cells by centrifugation for three minutes at 3000 rpm and then the final epithelial cell pellet was suspended in a small quantity of PBS and passed through 12µl Schisto kit filter.

The filters were placed face down on a microscopic slide and gently pressed to transfer the epithelial cells. The slides were dried, fixed with ethanol and Gram stained. Bacterial adhesion was determined by:

- Counting the number of adhered bacteria/100 buccal epithelial cells, and
- Counting the percentage of epithelial cells with adhered bacteria.

Both results have been done in the presence or absence of clove oil ^[16].

Antibacterial assay:

Screening of clove oil for antibacterial activity was done by the disc diffusion method ^[17]. It was performed using an 18 hours culture at 37°C in Muller - Hinton Broth. The cultures were adjusted to an approximately 105 cell/ml using an improved Neubauer Chamber.

Five-hundred microliter of the suspension was spread over the plates containing Muller-Hinton agar using a spreader in order to get a uniform microbial growth on test plates. The plates were allowed to dry under aseptic condition; empty sterilized discs were impregnated with 5µl, 10µl, 20µl & 30µl volume of the oil and placed on the agar surface. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then they were incubated at 37°C for 18 hours.

After the incubation period, the zone of inhibition was measured with a caliper ^[18]. Studies were performed in duplicate and mean value was calculated.

Determination of minimum inhibitory concentration, the MIC was defined as the lowest concentration that completely inhibited the growth of micro-

organisms. The MIC was determined by the micro dilution method. Clove oil was diluted.

A series of two fold dilution of clove oil ranging from 1/2 to 1/2042 was prepared in 5ml tryptic soy broth instead of using any chemical solvent which might have an antibacterial /anti-adherent activity ^[19,20].

These dilutions were mixed thoroughly before use. Bacterial inoculums were added to each tube. Inoculated tubes were incubated at 37°C for 18h and then MIC was determined. Experiments were carried out in duplicate. Inhibitions of bacterial growth in the tubes were judged by comparison with growth in the blank control tubes. Colony forming bacteria technique was done to check the growth in each tube. The MIC were determined as the lowest concentration (highest dilution) (v/v) of oil giving inhibition of visible growth of bacteria ^[21].

Result:

The result of this study showed that clove oil had an antibacterial activity against *S. aureus*.

Inhibition zone of growth above 7 mm was considered as a positive result ^[13].

Table (1) showed that zone of inhibition was increased with the increase of oil volume added to each disc and the average of two replicate was taken for each reading. The lowest concentration of clove oil which showed inhibition of *S. aureus* growth (MIC) was determined as it is shown in table (2). The dilution of 1/256 (0.391% v/v) was considered as the MIC for clove oil.

Table 1: Antimicrobial activity of clove oil using disc diffusion test (each value is average of two replicates)

Concentration of clove oil (µl/disc)	Diameter of zone of inhibition (mm)
5	11
10	13
20	17
30	19

Table 2: Minimum inhibition concentration (MIC) of Clove oil on *Staphylococcus aureus* growth
 *(MIC=1/256 which is equal to 0.391% v/v)

Dilution(V/V)	Results
1/2(50%)	No growth
1/4(25%)	No growth
1/8(12.5%)	No growth
1/16(6.25%)	No growth
1/32(3.125%)	No growth
1/64(1.563%)	No growth
1/128(0.781%)	No growth
1/256(0.391%)*	No growth
1/512(0.195%)	Positive growth
1/1024(0.098%)	Positive growth
1/2048(0.049%)	Positive growth

Dilution of 1/3(33.33% (v/v)) oil was selected to study its effect on prevention of *S. aureus* adhesion to buccal epithelial cells with the following results:

- a. Effect of clove oil on percentage of epithelial cells with adhered *S. aureus* as shown in figure 1 revealed that clove oil was reduced this percentage

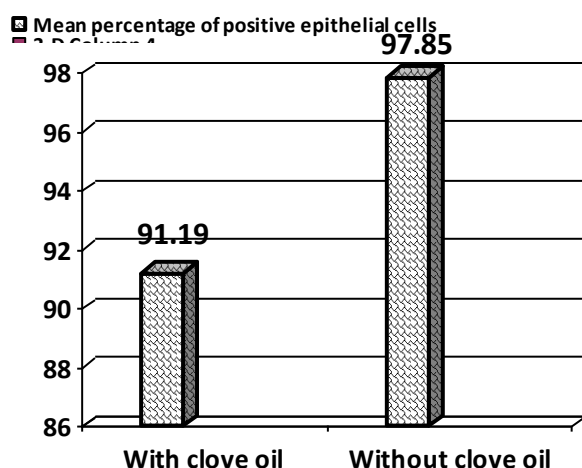


Fig.1: Percentage of positive epithelial cells (i.e. epithelial cells with adhered

from 97.78% (without oil) to 91.1% (with oil) with a P value ≥ 0.05 .

- b. Effect of clove oil on the mean number of *S. aureus* adhered to epithelial cell as shown in figure 2 showed that mean number of *S. aureus* adhered to epithelial cell was reduced from 116.76 to 12.55 with a P value of ≤ 0.05 .

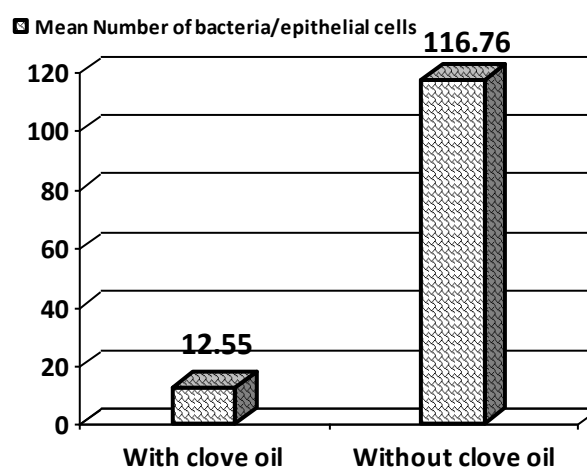


Fig. 2: Number of bacteria /epithelial cell

Discussion:

The antibacterial activities of clove oil were analyzed and clove oil showed a prominent antibacterial activity against *S. aureus* (Table 1). This result

generally matched the results obtained by Prabuseenivasan et al.^[17].

In a study carried out by Betoni et. al.,^[22]; also clove extract showed inhibitory effect against *S. aureus*.

Clove oil is known for its antibacterial activity which is due to several constituents and may be tested as an alternative to conventional antibiotics therapy^[23]. Cloves are strongly pungent due to their high content of eugenol, which is known to inhibit growth of Gram negative and positive and acid fast bacterium as well as fungi^[10].

Table (2) showed the results of the minimum inhibitory concentration of clove oil. Members of the clove oil components are known to be either bactericidal or bacteriostatic agents depending upon the concentration used (24).

These components were used without addition of any solubilizes agent such as dimethyl sulphoxide because some literatures such as Ansel et. al.,^[20] stated that the growth rate of some microorganisms were shown to be decreasing with increasing of DMSO concentrations, and electron microscopy revealed increased cytological alteration of micro-organism with increased concentrations of DMSO. Hili et.al.,^[19] proved and stated that there is an antagonistic effect of the solubilizes DMSO when it was mixed with cinnamon oil on microbial growth and this might be due to partitioning of the oil between the aqueous phase and DMSO distancing the oil from the cells. When no DMSO is used, the oil may be solubilized in the lipid membrane of the organism where it can have a greater effect on cell metabolism. Other literature^[19] reported that essential oil of clove dispersed (0.4%v/v) in a concentrated sugar solution ,had a marked germicidal effect against various bacteria and *Candida albicans* , strongly active, despite their low capacity to dissolve in water which is in agreement with published data^[24].

Clove oil appears to have potential use as botanical preservative.

The compounds with phenolic group such as eugenol are highly active against microorganisms. Clove oil has 79.2% eugenol. Gupta et.al.,^[18] and Cai & Wu^[13] listed a large group of active components in clove oil. Some workers have demonstrated potent antifungal, antiviral and antibacterial effects of clove oil^[11]. Prabuseenivasan et.al^[17] reported that clove oil was found to be equally effective

against both Gram positive and Gram negative organisms.

An important characteristic of essential oils and their components is their hydrophobicity which enable them to partition the lipid of the bacterial cell membrane and mitochondria disrupting the cell structure rendering them more permeable^[25,26]. Extensive leakage from bacterial cells or the exit of the critical molecules and ions will lead to death^[27].

1/3 oil dilution was chosen to determine its effect on *S. aureus* adhesion to buccal epithelial cells. The results revealed that percentage of epithelial cells with adhered *S. aureus* was decreased from 97.78% to 91.1% in the presence of clove oil (Figure 1), also the mean number of *S. aureus* cells per epithelial cell was reduced significantly from 116.76 to 12.55 with $P \leq 0.05$ (Figure2).

These results indicate that ability of bacteria to adhere to epithelial cells was reduced due to the presence of clove oil. There are several workers demonstrating that sub-inhibitory concentration of different antibiotics may interfere with processes of host pathogen interaction such as adherence^[28,29,30]. Bacterial adhesion represents the initial step in infection process and can be the result of either hydrophobia interactions between bacteria and host cell or binding of bacteria to specific ligands or combination of both^[31]. Stanley and Herschler^[32], stated that DMSO showed an inhibitory effect on NF-KB and intracellular adhesion molecules 1 gene expression in septic rat. Hili et al.^[19] stated that when DMSO is combined with another substance a new result was obtained which can exert a greater or lesser influence on a given process and concluded that it is essential in assessing the activity of the oil it should be agitated thoroughly with the broth in order to achieve full dispersal of the oil for good reproducibility of data and they showed in their study that much more activity (50 fold more) can be attained with cinnamon oil against *Saccharomyces cerevisiae* in the absence of solubilizes .Ghajar and Harmon (1968)^[33] studied the influence of DMSO on the permeability of *S. aureus* demonstrating that DMSO increased the oxygen uptake but reduced the rate of glycine transport. All of the above data indi-

cate that using of the solubilizing agents might have a definite effect on both antibacterial and adhesion assay, and for these reasons we used clove oil without solubilizing agent to study its pure effect on growth and adhesion of *S. aureus* on buccal epithelial cells in a pattern similar to that applied by the dentist to the patients.

For the best of our knowledge there is no published data studied the specific mechanism by which essential oils and their constituents interfere with the adhesion of this bacteria to human buccal cavity cells, and need to be studied. In conclusion clove oil was shown to have both antibacterial and anti-adhesion capabilities. These anti-adhesion activities beside of their previously known antibacterial activity are in favor of using this oil in oral treatment.

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