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Comparative studies on the effect of crude aqueous (CA) and solvent (CM) extracts of clove on the cariogenic properties of *Streptococcus mutans*

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Abstract: A study was conducted to compare the efficiency of crude aqueous (CA) and solvent extracts (CM) of clove on the caries-inducing properties of *Streptococcus mutans*. The cariogenic properties investigated included the cell adhesion, cell-surface hydrophobicity and glucan synthesis activities of *S. mutans*. There was a significant difference between the effect of the CA and CM extracts on the adhesion of *S. mutans* ($P < 0.05$) within a concentration range of 5-15 mg/ml, the CM extract demonstrating a slightly higher inhibitory effect. However, the effect of the CM extract on the cell-surface hydrophobicity of *S. mutans* was weaker than that of the CA extract. The two extracts were found to reduce the synthesis of water-insoluble glucan (WIG) by almost 50% at a concentration as low as 0.5 mg/ml and the CM extract exhibited a significantly higher inhibitory effect than the CA extract ($P < 0.05$). The present findings indicate that both the CA and CM extracts exert inhibitory effects on the cariogenic properties of *S. mutans* and that the CA extract is as equally effective as the CM extract. (J. Oral Sci. 48, 117-123, 2006)

Keywords: clove; *Streptococcus mutans*; anti-adhesion; cell surface hydrophobicity; glucan synthesis.

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Introduction

In many parts of the world there is a rich tradition in the use of natural products for the treatment of many infectious diseases. Natural products have been used for thousand of years in traditional medicine for many purposes. Many herbal remedies have been used because of their anti-bacterial, anti-inflammatory, cytostatic (1), anti-fungal and anti-viral activities. The World Health Organization estimates that 80% of people living in developing countries use traditional medicine almost exclusively (2,3).

Cloves are the dried, unopened inflorescence of the clove tree, *Syzygium aromaticum*, which is a member of the Myrtaceae family. Cloves vary in length from about 1/2 to 3/4 inch and contain 14-20% essential oil. Cloves are strongly pungent due to their high content of eugenol, which can be extracted by distillation to yield the essential oil. Clove buds have been regarded as safe when taken orally for medicinal use. Cloves have been used by humans for medicinal applications for over two thousand years, being chewed to alleviate the pain of toothache, and are also widely used to disinfect root canals in temporary fillings (4) and as an oral anesthetic. Eugenol is a chemical compound present in cloves, and is known to inhibit the growth of bacteria. It is a natural antibiotic (5) with broad antimicrobial activities against gram-positive, gram-negative and acid-fast bacteria, as well as fungi (6,7).

Oral bacteria play an important role in the formation of dental caries and development of 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. *Streptococcus mutans* is an oral bacterium known to be

aciduric and acidogenic (8). It has been implicated as one of the primary causative agents of dental caries in humans and experimental animals (9). Controlling the growth of *S. mutans* may limit the formation of cariogenic dental plaque.

The caries-inducing properties of *S. mutans* depend on its adhesion ability (10), and also its acid-producing and glucan synthesis activities. Bacterial adhesion or attachment to the tooth surface involves specific molecular interactions between complementary molecules on the microbial and host surface, which require the participation of two factors; a receptor and adhesin. Hydrophobic forces or cell surface hydrophobicity also play an important role in the initial adhesion of bacteria to tooth surfaces (11). Among the oral streptococci, *S. mutans* has a higher degree of surface hydrophobicity (12), and its adhesion to saliva-coated hydroxyapatite is dependent on hydrophobic interaction (13).

S. mutans produces glucosyltransferase (GTF), which allows for the production of soluble and insoluble glucan. Glucosyltransferase from *S. mutans* is the most significant virulent factor in dental caries, where sucrose plays an important role as a natural source of energy and is converted to long-chain polysaccharides (9,14,15).

Currently, there is great concern about environmental pollution due to solvents that are employed in the preparation of plant extracts. To minimize this, an alternative preparation of the extract employing an environmentally friendly procedure has been considered. In traditional medicine, plant extracts have always been prepared using decoctions of aqueous extracts (16-18). In this study, we investigated and compared the effects of crude aqueous and solvent extracts of cloves on the caries-inducing properties of *S. mutans*.

Materials and Methods

Plant materials

Cloves buds were bought from the local market in Kuala Lumpur.

Chemicals

The chemicals used were: brain heart infusion broth (BHI) (Oxoid, Hampshire, England); glycerol, sucrose, sodium chloride, ethanol (BDH Laboratories Supplies, Poole BH15 ITD, England); n-hexadecane, exogenous dextran (ICN Biomedicals INC., OH, USA); sodium phosphate (Sigma Chemicals Co., MO, USA); glucose, merthiolate (Sigma Aldrich Co., MO, USA); and methanol (Fisher Scientific, Leicestershire, UK).

Preparation of bacterial suspension

The *S. mutans* ATCC 25175 strain was bought from ATCC, USA. A stock of *S. mutans* was prepared in glycerol and kept at -80˚C until further use. To revive the *S. mutans* strains, the stock was thawed and inoculated into BHI. The inoculated broth was then incubated for 18-24 h at 37˚C. For this study, the bacterial suspension used was adjusted to an optical density of about 0.144 at 550 nm wavelength, and this was equivalent to 10^6 cells (19).

Preparation of the crude aqueous and solvent clove extracts

Aqueous extract

One hundred grams of clove powder was boiled in 1 liter of deionised distilled water (ddH_2O) and allowed to boil to a final volume of 100 ml. The concentrated mixture was filtered and the clear extract was aliquoted in 1-ml volumes into Eppendorff tubes. The extracts were dried overnight using a speed vacuum concentrator until no further changes in weight were observed. The dried extracts were then stored at -20˚C until further use.

Solvent extract

Clove powder (100 g) was put in a pre-weighed flask and extracted with methanol for 16 h. The extract collected was evaporated to dryness using a rotary evaporator in vacuum at 60˚C. The flask was reweighed and the difference in the weight gave the weight of the extract collected. The dried extract was then dissolved in methanol and aliquoted in 1-ml volumes into Eppendorff tubes. The extracts were then dried and stored for further use according to the procedure described for the aqueous extracts.

Determination of the *in vitro* effect of clove extracts on cell adhesion of *Streptococcus mutans*

S. mutans culture was grown in a test tube containing 3 ml of BHI broth with 1% (w/v) sucrose at 37˚C and held at an angle of 30˚ for 18 h. After 18 h incubation, the adhering cells were collected by sonicating and washing the tube with saline (0.9% NaCl), and then determined turbidimetrically at $OD_{550\ nm}$. This gave the total cell number and represented 100% adhesion. The experiment was repeated by incubating the *S. mutans* culture in 3 ml BHI broth with 1% (w/v) sucrose and different concentrations [5-20 mg/ml (w/v)] of crude clove extracts (CA and CM). This gave the total cell number adhering in the presence of different concentrations of the extract, and this was expressed as a percentage of the total cell number in the absence of the extract.

Determination of the *in vitro* effect of clove extracts on cell surface hydrophobicity of *Streptococcus mutans*

S. mutans cultures were grown in 200 ml of BHI broth for 18-24 h and collected by centrifugation. The cells were washed twice and suspended in 1 ml saline [0.9% (w/v)], giving an absorbance at 550 nm equivalent to 1.2. The cell suspension was then mixed with an equal volume of ddH_2O. The suspension was allowed to stand for 10 min at room temperature before the optical density of the suspension was determined at 550 nm. This gave the total number of cells in the aqueous phase before agitation. Subsequently, 0.2 ml of n-hexadecane was added and the suspension was agitated uniformly using a vortex mixer for 1 min. After the n-hexadecane phase had separated from the aqueous phase, the optical density of the aqueous phase was determined at 550 nm. This gave the total number of cells in the aqueous phase after agitation.

The percentage of hydrophobic cells was measured using the formula:

% of hydrophobic cells = [(total number of cells in aqueous phase before agitation - total number of cells in aqueous phase after agitation) / total number of cells in aqueous phase before agitation] $\times$ 100%

The experiment was repeated by mixing the cell suspension (1 ml) with an equal volume of different concentrations of crude clove extracts (CA and CM) [5-20 mg/ml (final concentration)] in the place of ddH_2O.

Determination of partially purified crude cell-associated and cell-free glucosyltransferase activities of *Streptococcus mutans*

Determination of the enzyme activities was based on the amount of glucans formed, expressed as the glucose content in one minute. The reaction mixtures used for determination of the partially purified cell-associated glucosyltransferase (CAG) and crude cell-free glucosyltransferase (CFG) activities were the same, and a modification of the method described by Mukasa et al. (20) was employed. The reaction mixture was composed of 0.01% merthiolate, 1.4 g of sucrose, and 34.3 mg of exogenous dextran in 100 ml of 5 mM sodium phosphate buffer (pH 6.5).

i) CAG activity

One hundred microliters of the partially purified crude CAG was pipetted into the reaction mixture and made up to a final volume of 2 ml. The mixture was then incubated for 18 h at 37˚C, and the reaction was terminated by placing the tube in a boiling water bath for 5 min. The mixture was then centrifuged at 2,000 $\times$ g for 10 min, and the supernatant was discarded. The pellet obtained was washed three times with 5 ml of distilled water, smoothly suspended by agitation in 1 ml of distilled water, and used for determination of the water-insoluble glucan content.

ii) CFG activity

The procedure was repeated using the partially purified crude CFG in the place of partially purified CAG. The pellet obtained was discarded, the supernatant (1 ml) obtained was collected and used for determination of the water-soluble glucan content.

Quantitation of water-insoluble and water-soluble glucan

The quantitation of the glucans was based on the glucose content. For the quantitation of water-insoluble glucan, 50 μl of the suspension obtained as described in i) was used in the assay for glucose content.

For the quantitation of water-soluble glucan, 1 ml of the supernatant obtained as described in ii) was precipitated with four volumes of absolute ethanol. The precipitate formed was then washed three times with 5 ml of 75% ethanol and subsequently dissolved in 1 ml of distilled water. 50 μl of this was then used in the assay of glucose content.

The glucose content was determined according to the method described by Dubois et al. (21). The enzyme activities were calculated and expressed as μmol glucose content in glucan produced per minute (μmol/min). The assay was carried out in triplicate.

Determination of the effect of crude clove extracts on partially purified crude CFG and CAG activities

The reaction mixture used in the above experiment was incubated with various concentrations of the clove extract for 18 h at 37˚C. The amount of water-insoluble and water-soluble glucan formed was then determined according to the procedure described above. The amount of glucan (expressed in μmol glucose) formed per minute per mg protein was considered to represent the specific activities of the enzymes.

Statistical analysis

For cell adhesion, and cell surface hydrophobicity, data obtained for the test and the control groups were compared using ANOVA (Two Factors with Replication, using Excel). The cell adhesion and cell surface hydrophobicity were compared between the CA and CM extracts and the CAG and CFG activities using *t* test. All of the experiments were carried out in triplicate and repeated three times.

Results

Figure 1 shows the effect of CA and CM extracts on the adhesion of *S. mutans* to a glass surface. It was found that the CA and CM extracts had inhibitory effects on the adhesion of *S. mutans*, the CM extract exhibiting a stronger inhibitory effect than the CA extract. The inhibitory effect increased with increasing extract concentration. At a concentration of 5 mg/ml, the percentage of adhesion due to the CA and CM extracts was reduced to 83.9 ± 9.1% and 63.8 ± 6.7%, respectively. At 10 mg/ml, the CM extract exhibited almost 100% inhibition compared to the CA extract (48%). Complete inhibition (100%) was observed upon exposure to 15 mg/ml of CM extract. For the CA extract, a higher concentration (20 mg/ml) was required to cause 100% inhibition.

The inhibitory effect due to the different concentrations of the respective CA and CM extracts compared to the blank control (ANOVA) was significant ($P < 0.05$). Comparison (*t* test) between the effect of CA and CM at 5 mg/ml, 10 mg/ml and 15 mg/ml showed significant differences ($P < 0.05$), but there was no significant difference between the effect of the CA and CM extracts at 20 mg/ml.

Figure 2 shows the effect of the CA and CM extracts on the cell surface hydrophobicity of *S. mutans*. Both extracts reduced the cell surface hydrophobicity of *S. mutans* and the effect increased with increasing extract concentration. However, the CM extract was less effective than the CA extract. The cell-surface hydrophobicity of *S. mutans* in the absence of the extract was about 70.8 ± 5.0%. In the presence of the CA and CM extracts at 5 mg/ml, the cell-surface hydrophobicity was reduced to 18.8 ± 7.1% and 63.9 ± 4.8%, respectively. Exposure of *S. mutans* to a higher concentration of the CA and CM extracts (20 mg/ml) reduced the cell surface hydrophobicity even further to about 0.3 ± 0.1% and 25.2 ± 4.7%, respectively. Comparison between the CA and CM extracts at concentrations of 5, 10, 15 and 20 mg/ml (*t* test) showed that their effects were significantly different ($P < 0.05$).

Figure 3 shows the effect of the crude clove extracts on the activity of CAG. The CM extract reduced the specific activity of CAG much more effectively and significantly when its concentration was increased to 2.5 mg/ml, compared to the CA extract at the same concentration. Fig. 4 shows the effect of the extract on the specific activity of CFG. The CA extract demonstrated a much stronger inhibitory effect, but was not significantly different from that of the CM extract.

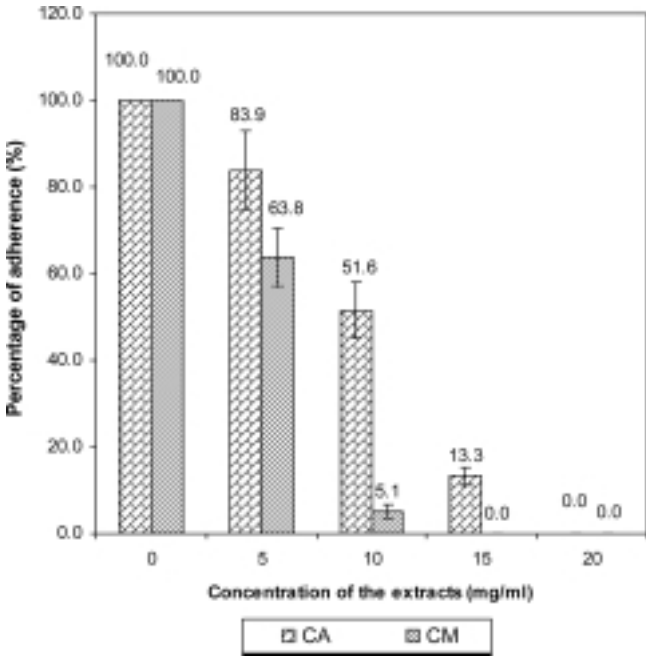

Fig.1 Effect of crude clove extracts (CA and CM) on the cell adhesion of *Streptococcus mutans* ATCC 25175 strain. Concentration (mg/ml) refers to the assay that was carried out in the absence of the extract and represents the blank control. The data were expressed as means ± standard deviation (SD) of 9 determinations.

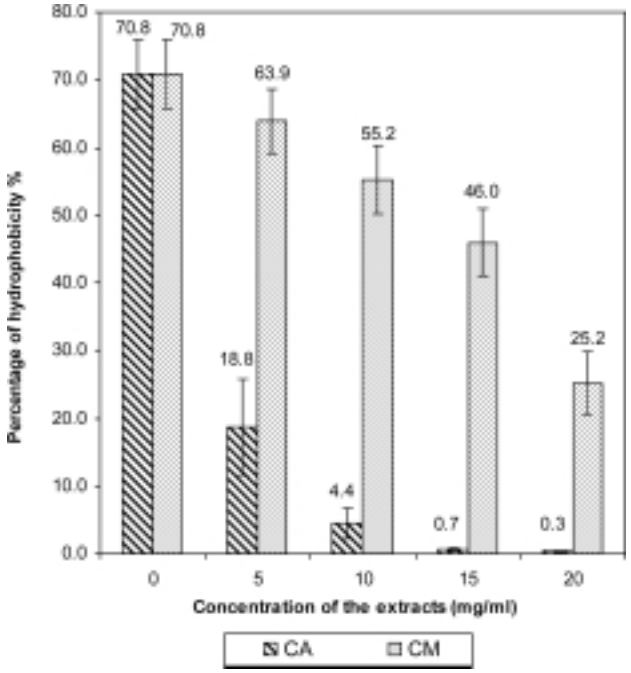

Fig. 2 Effect of crude clove extracts (CA and CM) on cell-surface hydrophobicity of *Streptococcus mutans* ATCC 25175 strain. 0 mg/ml refers to the assay that was carried out in the absence of the extract and represents the blank control. The data were expressed as means ± standard deviation (SD) of 9 determinations.

Discussion

It was observed that the ability of *S. mutans* to adhere to a glass surface was affected by the presence of crude clove extracts, the CM extract exerting slightly stronger inhibition than the CA extract. The anti-adhesion effect was initially thought to be due to the antimicrobial activity of eugenol towards *S. mutans*. However, matching of the HPLC fractions of the extract with antimicrobial activity did not support this. It has been reported that inhibition of bacterial adhesion could be due to alteration of the initial attachment of *S. mutans* (22,23). The receptors on the cell surface of *S. mutans* may be modified by components in the crude CM and CA extracts, leading to reduction of adhesion ability. The adhesion was reduced to 63.8 ± 6.7% at 5 mg/ml CM extract, and a higher concentration of the CA extract (10 mg/ml) was required for a similar reduction (51.6 ± 6.6%). This implied that the active component(s) are present in the CA as well as the CM extract, being slightly more potent in the methanol than in the aqueous extract. The reduced adhesion of microbial cells to the tooth surface would disrupt the colonization of the tooth surface by the microorganisms, and this could affect plaque accumulation.

Krepsky et al. (24) have suggested that hydrophobicity is one of the important properties allowing microorganisms to adhere to both living and non-living surfaces. *S. mutans* is more hydrophobic than other streptococcus species. Hence, changes in cell surface hydrophobicity may affect bacterial cell adhesion ability. In this study, it was shown that despite reducing the cell surface hydrophobicity of *S. mutans* to a lesser degree than the CA extract, the CM extract exhibited a much greater anti-adhesion effect. This suggests that the cell surface hydrophobicity of *S. mutans* does not play an important role in bacterial adhesion. This appears to reflect the observation reported by Katsikogianni and Mirssirlis (25) that material surface hydrophobicity plays a more important role than bacterial surface hydrophobicity.

To sustain a thicker plaque, bacteria need to synthesize the extracellular glucan or polysaccharides that form the extracellular matrix of plaque. Thicker plaque will create a compact and anaerobic environment. The formation of polysaccharides or glucan is catalysed by glucosyltransferase (9,14). The CA and CM extracts were observed to inhibit the activity of CAG. The rate of formation of this polysaccharide was inhibited by 50% in

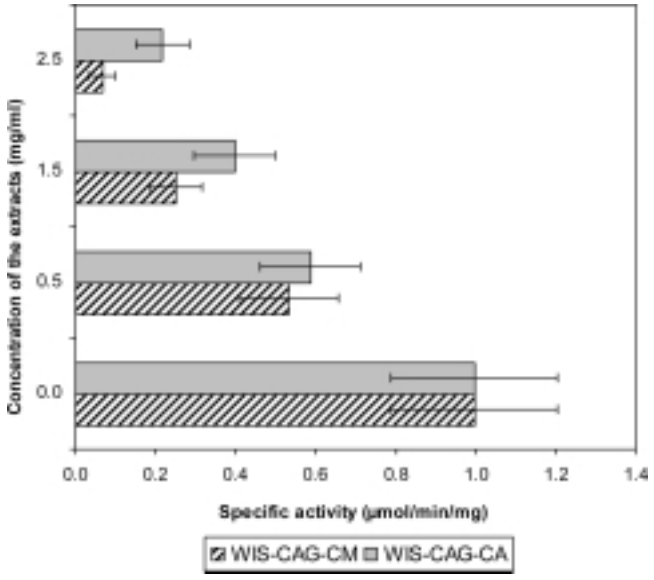

Fig. 3 Effect of crude aqueous clove extract (CA) on the specific activity of CAG (WIS-CAG-CA) and crude solvent clove extract (CM) on the specific activity of CAG (WIS-CAG-CM). 0 mg/ml refers to the assay that was carried out in the absence of the extract, and represents the blank control. The *P* value indicated a significant difference ($P < 0.05$) between the specific activities of CAG in the presence of the CA extract compared with the CM extract. The data were expressed as mean ± standard deviation (SD) of 9 determinations.

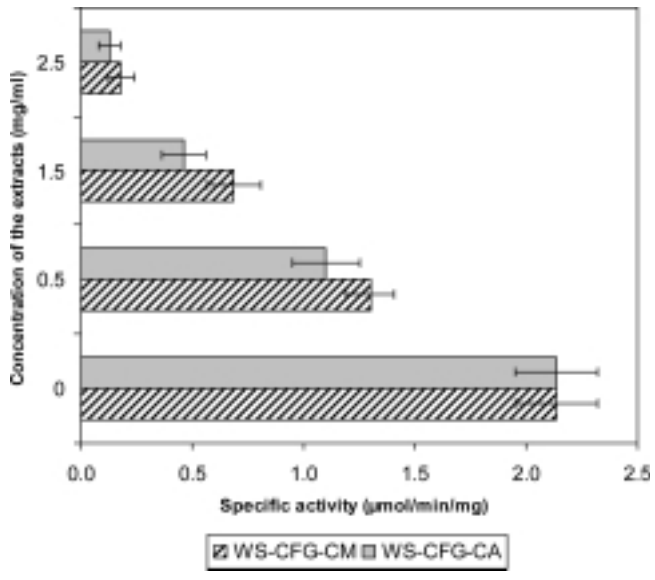

Fig. 4 Effect of crude aqueous clove extract (CA) on the specific activity of CFG (WS-CFG-CA) and crude solvent clove extract (CM) on the specific activity of CFG (WS-CFG-CM). 0 mg/ml refers to the assay that was carried out in the absence of the extract, and represents the blank control. Results are expressed as the mean value ± standard deviation (SD) of 9 determinations.

the presence of 0.5 mg/ml extract. Comparison of the inhibitory effect between the two extracts showed no significant difference at lower concentrations (0.5 and 1.5 mg/ml) and a significant difference at a higher concentration (2.5 mg/ml). This suggests that at lower concentrations, similar components present in the CA and CM extracts cause the inhibitory effect, and at a higher concentration, other components in the CM extract not present in the CA extract exhibit an additive effect. The inhibitory effect on the formation of extra-cellular polysaccharides may indicate that the crude clove extracts possess components that have potential anti-plaque properties, and consequently an anti-caries effect. Other studies using different plants (18,26,27) have also produced similar findings. Future studies should aim to identify the components in the CA and CM extracts.

Studies carried out by Wu-Yuan et al. (28) have shown that extracts from the plant *Melaphis chinensis* exert an inhibitory effect on the synthesis of water-soluble glucan by inhibiting the activity of CFG. Although the role of water-soluble glucan in dental plaque formation has been less emphasized compared to that of cell adhesion and water-insoluble glucan, it nevertheless contributes to the energy reserves of *S. mutans* strains. The inhibitory effect is concentration-dependent, increasing with higher concentration.

The results obtained from these *in vitro* studies have demonstrated that crude clove extracts (CA and CM) may have the potential to influence the plaque-inducing properties of *S. mutans* ATCC 25175 strain by affecting cell adhesion, cell-surface hydrophobicity and GTF activities. This will subsequently affect the caries-inducing properties of the bacterium. The degree of effectiveness of the CA and CM extracts, however, differs within a narrow range, making the two extracts equally effective.

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