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Volatile Components of *Camellia sinensis* Inhibit Growth and Biofilm Formation of Oral Streptococci *in vitro*

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Abstract: This study aimed to evaluate the efficacy of semi fermented and non fermented *Camellia sinensis* extracts (Black and Green tea) and comparison between them against *Streptococcus mutans* ATCC 25175, *S. mitis* ATCC 9811 and *S. sanguis* ATCC 10556 that are responsible for dental caries and bacteremias following dental manipulations. Minimum inhibitory concentrations of both tea extracts were assessed by Well diffusion and Broth dilution methods and examination of cell adherence (Biofilm inhibitory concentrations) was observed on glass slides under phase contrast microscope and colony counts from glass beads. Concentration of 1 mg mL⁻¹ of semi fermented tea extract was completely biofilm inhibitor but biofilm formation by these bacteria was seen 7 days after treatment with 1 mg mL⁻¹ of non fermented *Camellia sinensis* on glass beads and BIC for oral streptococci treated with this extract was 1.5, 2.5 mg mL⁻¹ of semi fermented and 3 mg mL⁻¹ of non fermented extracts had bactericidal effect on these bacteria. Semi fermented and non fermented *Camellia sinensis* extracts were able to prevent growth of oral streptococci. Therefore dental caries significantly reduce and the efficiency of semi fermented tea was higher due to rich content of volatile components rather than non fermented extracts.

Key words: Semi fermented, non fermented, *Camellia sinensis*, dental caries, tea components, biofilm

INTRODUCTION

The two most common types of dental diseases, dental caries and periodontal disease, are plaque-related infections. Dental caries involves demineralization, cavitation and breakdown of calcified dental tissue and is caused by microorganisms that ferment dietary carbohydrates, notably sucrose, to produce acids; these acids initiate dissolution of the tooth enamel. Strains of *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus sanguis* have long been implicated in the formation of dental plaque and cariogenicity and are often responsible to bacteremias following dental manipulations (Tomás *et al.*, 2005). Natural products are now preferred by a large proportion of the population and have been reported to possess antimicrobial activity (Hammer *et al.*, 1999). Plant extracts have recently been shown to be a good alternative to synthetic chemical substances for caries prevention (Hamilton-Miller, 1995). Extracts from *Celastrus scandens*, *Chamaebatia foliolosa*, *Digitaria sanguinalis*, *Ginkgo biloba*, *Juniperous virginiana* (Heisey and

Gorham, 1992), *Anacardium occidentale* L. (Muroi and Kubo, 1993) and *Ilex paraguayensis* (Kubo *et al.*, 1993) have proved to be effective against *Streptococcus mutans*.

Tea is prepared from the young shoots of tea plant *Camellia sinensis*. Five thousand years ago Chinese identified this plant and used it for patient treatment and staining textile (Koo and Cho, 2004). Extracts of leaves from the tea plant *Camellia sinensis* contain polyphenolic components with activity against a wide spectrum of microbes. Studies conducted over the last 20 years have shown that the green tea polyphenolic catechins can inhibit the growth of a wide range of Gram-positive and Gram-negative bacterial species with moderate potency. Toda *et al.* (1989) found that extract of tea inhibited and killed *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhi*, *S. typhimurium*, *S. enteritidis*, *Shigella flexneri*, *Shigella dysenteriae* and *Vibrio* species including *Vibrio cholerae*. Tea extracts have been found to be active against *Clostridium* spp. and phytopathogens such as *Erwinia* spp. and *Pseudomonas* spp. (Ahn *et al.*, 1991).

The aim of this study was to evaluate the antimicrobial activity and bactericidal concentrations of non fermented and semi fermented *Camellia sinensis* extracts (green and black tea) on oral Streptococci and compare their effects on biofilm formation and adherence to surfaces using a variety of *in vitro* test systems.

MATERIALS AND METHODS

Extraction of samples: Fresh grade 1 black (semi fermented) and green (non fermented) tea that were essence free, quickly after production purchased from traditional producer company in Lahijan city in the north of Iran. Tea samples were stored in plastic bags at 4°C and transported to the microbiology laboratory of Science and Research campus of IAU in Tehran. Samples powdered in a blender and then extracted with methanol: water mixture (62.5:37.5 v/v) in the Clevenger extractor as follows: powdered dry material (500 mg) was weighed into a test tube. A total of 10 mL of 62.5% aqueous methanol was added and the suspension was stirred slightly. Tubes were sonicated 5 min and centrifugated for 10 min (1500 g) and supernatants were collected. The materials were re-extracted twice. The extracts were concentrated by further evaporation to one-fifth volume, filter sterilized and stored at 4°C.

Gas chromatography: GC analyses were performed with a Shimadzu 17A gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector and a 60 m × 0.25 mm (I.D.) DB-WAX (J and W Scientific, Folsom, CA) fused-silica capillary column. The operating conditions were as follows: oven temperature, 40, 3°C min⁻¹ rise to 220°C; carrier gas, helium and flow rate, 1.0 mL min⁻¹. The retention times and peak areas of the eluted volatile components were integrated with a Chromatopack integrator.

GC-MS analysis: GC-MS analysis was carried out on a Hitachi M-808 double-focusing instrument equipped with a Hewlett-Packard 5890 gas chromatograph. The GC conditions were identical to those of the above analytical GC runs. Mass spectral data were acquired and processed by a built-in computer system (M-0101) developed by Takasago International. The components of the distillate were identified by comparing their GC retention times and MS fragmentation with those of the authentic samples.

Bacterial strains and cultures: *Streptococcus mutans* ATCC 25175, *Streptococcus mitis* ATCC 9811 and *Streptococcus sanguis* ATCC 10556 that purchased from

Microbiological Reference Centre of Iran, were grown on brain heart infusion broth, (BHI Difco, Detroit, USA) at 37°C with 5% defibrinated sheep blood in an atmosphere contain 5% CO₂ and for antimicrobial susceptibility test, Mueller Hinton Agar (MHA, Difco, France) was used.

Antimicrobial activity tests: For this examination Well diffusion and broth dilution methods were used separately. Standardized bacterial suspensions containing 10⁶ cells mL⁻¹ were obtained by spectrophotometry (Shimadzo UV 120-01), then 100 µL of this suspension cultured onto Mueller Hinton Agar with cotton Swab, then 30 µg of black and green tea extracts (100 mg mL⁻¹) were added separately to 5 mm wells in a Mueller Hinton Agar supplemented with 5% defibrinated sheep blood and maintained 48 h in 37°C incubator with 5% CO₂ atmosphere and then their MIC were assessed. Ten microgram discs of Penicillin used for positive control and distilled water used for negative control.

In broth dilution method 50 µg of black and green tea extracts added to 10 mL BHIB containing 5% defibrinated sheep blood that inoculated with 10⁶ cells mL⁻¹ and incubated in 5% CO₂. After 5 min of contact at room temperature, surviving Colony Forming Units (CFU) were counted by inoculating of 0.5 mL treated sample cultures on BHI agar at 37°C with 5% defibrinated sheep blood in an atmosphere contain 5% CO₂ after 48 h. Each experiment was carried out twice, duplicates and controls with no black and green tea extracts were always included.

Bacterial adherence to glass beads: Nine milliliters mixtures of each tea extract (Semi fermented and non fermented Persian *Camellia sinensis* at concentration of 1, 2 and 3 mg mL⁻¹) with dehydrated culture medium (BHI broth) were distributed into 15 tubes. Then, standardized glass slides (diameter = 2 mm, length = 5 cm) and glass beads (Spherical beads with 5 mm diameter) were added to the separate tubes and submitted to autoclave sterilization. For evaluation of bacterial adherence, one milliliter of standardized bacterial suspension (10⁶ cfu mL⁻¹) obtained by spectrophotometry was inoculated into each tube (final volume 10 mL) and then incubated for 90 min at 37°C in atmosphere of 5% CO₂. After the period of incubation, the glass slides and glass beads were transferred into tubes containing buffered phosphate saline pH 7.2 (PBS, Sigma, USA). Tubes containing glass beads were submitted to agitation (Phoenix AP56, Brazil), from this initial suspension; dilutions of 10⁻¹ and 10⁻² were obtained in sterilized NaCl 0.85% saline solution. Then, aliquots of 0.1 mL of each dilution were plated in

duplicate on BHI agar and incubated for 48 h at 37°C in atmosphere of 5% CO₂. After this period, the number of colonies was counted and the value of logarithm of colony forming units per milliliter was calculated (log cfu mL⁻¹). The results obtained were analyzed statistically by ANOVA test (p<0.01).

On the other hand, the glass slides that obtained from control tube and treated with different concentration of tea extracts were directly examined under phase contrast microscopy and the adherence pattern of the treated and non treated bacteria was observed and compared with each other.

RESULTS

GC mass spectroscopy results: Extracts of non-fermented (green) and semi-fermented (Black) teas prepared from *Camellia sinensis* were compared by means of GC (Table 1). The amounts of almost all volatile components, including alcoholic aroma constituents hydrolyzed by enzyme increased as a result of semi-fermentation.

Antimicrobial activity: In comparison between semi and non fermented tea shoots, growth inhibitory concentration was lower for semi fermented *Camellia sinensis* extract and its antimicrobial activity was better as compared to green tea. The diameter of inhibitory zone in concentration of 3 mg mL⁻¹ of black tea extract was 25.5 mm for *Streptococcus mutans*, 28 mm for *Streptococcus mitis* and 29.5 mm for *Streptococcus sanguis* and for the same concentration of green tea extract the MICs were 24, 26.5 and 27.5 mm orderly. This is a noticeable result if compared with positive control (Penicillin) that has 33, 34 and 33 mm inhibitory zone in order for these bacteria. The results are shown in Table 2.

Bactericidal effect of both extracts was clear and significant different exist between extracts effects. In comparison between non fermented and semi fermented *Camellia sinensis* extracts, the later has faster effect on inhibition growth of oral streptococci. In case of 3 mg mL⁻¹ of black tea extract after 30 min the count of viable cells was below 1 log of cfu mL⁻¹ and this amount achieved by same amount of green tea extract in 40 min (Fig. 1-3).

Oral Streptococci's adherence to glass: ANOVA test results showed significantly higher counts for control group (p<0.01) and the lowest counts were obtained for semi fermented *Camellia sinensis* extract (p<0.01) (Fig. 4).

Table 1: The content of volatile components in Persian (Lahijan) Black and Green teas (*Camellia sinensis*)

Compound	Green tea (mg g ⁻¹) (Non fermented)	Black tea (mg g ⁻¹) (Semi fermented)
Heptanal	0.72	2.72
Hexanal	0.05	0.32
Pentanol	0.11	1.07
Hexanol	0.13	0.58
Nonanal	0.70	2.18
Linalool oxide (cis furanoid)	0.09	0.19
Linalool oxide (trans furanoid)	0.08	0.09
Linalool	0.41	2.00
Octanol	0.19	0.44
Hexenyl hexanoate	0.08	1.64
Linalool oxide (trans pyranoid)	0.10	0.30
Geraniol	0.11	0.44
Benzyl alcohol	0.08	0.19
Beta ionone	0.08	0.40
Jasmone	Trace	0.43
Nerolidol	0.11	0.83
Methyl anthranilate	0.11	0.19
Indole	0.01	0.45

The content of each component expressed by the ratio of each peak area of the internal standard of the gas chromatogram

Table 2: *In vitro* susceptibility of *S. mutans* ATCC 25175, *S. mitis* ATCC 9811 and *S. sanguis* ATCC 10556 to semi fermented and non fermented *Camellia sinensis* extracts

Strains	Inhibitory zone (mm)		
	Semi fermented <i>C. sinensis</i> (3 mg mL ⁻¹)	Non fermented <i>C. sinensis</i> (3 mg mL ⁻¹)	Penicillin (10 µg mL ⁻¹)
<i>S. mutans</i> ATCC 25175	25.5	24.0	33
<i>S. mitis</i> ATCC 9811	28.0	26.5	34
<i>S. sanguis</i> ATCC 10556	29.5	27.5	33

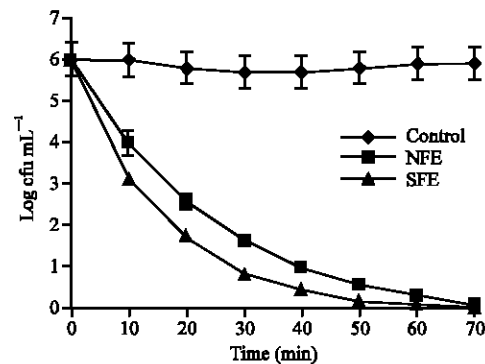


Fig. 1: Growth inhibition of *Streptococcus mutans* ATCC 25175 to semi fermented *Camellia sinensis*

Phase contrast microscopy: Microscopic examinations showed that untreated oral streptococci were able to attach each other and produce a clump on the slide surface (Fig. 5) that is a basic and initial step for biofilm formation but 1 mg mL⁻¹ of semi fermented and 1.5 mg mL⁻¹ of non fermented *Camellia sinensis* extracts inhibited the attachment of these bacteria and bacterial

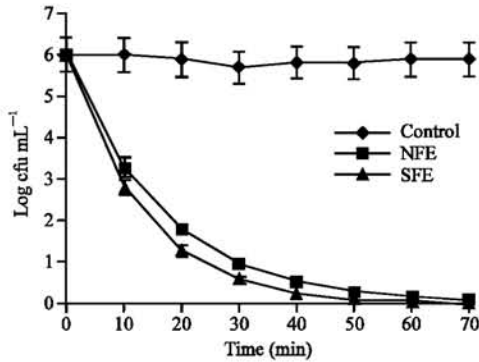


Fig. 2: Growth inhibition of *Streptococcus sanguis* ATCC 10556 to semi fermented *Camellia sinensis*

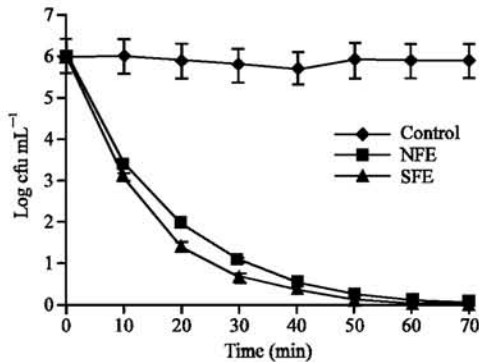


Fig. 3: Growth inhibition of *Streptococcus mitis* ATCC 9811 to semi fermented *Camellia sinensis*

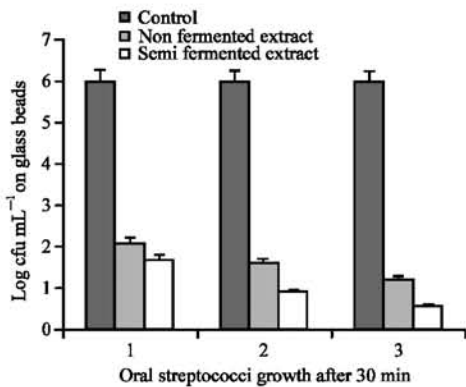


Fig. 4: The numbers of oral streptococci treated with 2 mg mL⁻¹ of non fermented and semi fermented *Camellia sinensis* on glass beads. The bacterial strains shown with (1) *S. mutans*, (2) *S. mitis* and (3) *S. sanguis*

cell were washed easily and crowded on some areas. This result confirms biofilm inhibitory effects of these extracts especially semi fermented extract.

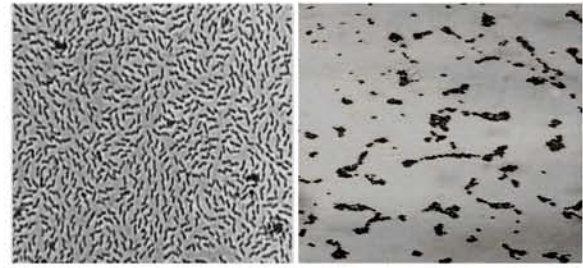


Fig. 5: Phase contrast microscopy images of *Streptococcus mutans* ATCC 25175 before treatment (Left image) and after treatment with 1 mg mL⁻¹ of semi fermented *Camellia sinensis* extract (Right image) taken from glass slide surfaces

DISCUSSION

Recent investigations are focusing on the nutritional effects of tea on the human body and health. In this study among the two types of tea extracts that tested on oral streptococci, semi fermented *Camellia sinensis* (black tea) seems to be the more effective extract against streptococcal growth and biofilm formation (Table 2). Epidemiological studies revealed a reduction in caries formation in tea drinking populations and school kids from tea planted areas in Japan (Onisi, 1993).

Black tea has many more components than green tea, to some extent because of the oxidation processes that occur during fermentation. Further reactions take place when the dried finished tea leaves are extracted into water, increasing the complexity of the chemical mix in a cup of tea. In addition, further chemical changes occur when a cup of tea is left to stand. Results of GC-MS shows much higher amounts of volatile component in *Camellia sinensis* after exposure to fermentation (Table 1). Volatile flavor components make up a very small fraction of flush and tea leaf (10 to 20 ppm) but play an important part in providing taste and antibacterial activity in our study. More than 300 such components have been reported in black tea leaf (Gutman and Ryu, 1996) and more than 100 such components have been reported in green tea. Kubo *et al.* (1993) found some of these volatile components to be microbiologically active, but not at cup-of-tea concentrations (Ahn *et al.*, 1990).

Minimum inhibitory concentrations and Biofilm inhibitory concentrations of semi fermented *Camellia sinensis* were lower for tested oral streptococci in comparison to non fermented *Camellia sinensis* (Table 2) that shows a direct relationship with amount of volatile components of extracts (Table 1). Green tea consumption

has been reported to increase the acid resistance of teeth to damage by cariogenic bacteria (Gutman and Ryu, 1996; Hamilton-Miller, 2001). It has been demonstrated that tea can inactivate glucosyltransferase and dextran sucrose, thus it can inhibit the formation of water insoluble glucan and lactic acid, respectively (Otake *et al.*, 1991).

Among the studied oral bacteria, *S. mutans* ATCC 25175 was more resistant (Fig. 1) and *S. sanguis* ATCC 10556 was more sensitive to the both tea extracts (Fig. 2).

Combinations of the flavor compounds, especially indole with some of the sesquiterpenes, displayed marked bactericidal synergy (Hamilton-Miller, 1995). Bacterial adherence to glass surface is the model system chosen because the adherence is mediated by glucan as well as the *in vivo* situation and the glass adherence assay is still used in some recent studies (Koo *et al.*, 2000; Mattos-Graner *et al.*, 2000; Carter *et al.*, 2001; Tao and Tanzer, 2002).

The numbers of oral streptococci cells that treated with 2 mg mL⁻¹ of non fermented and semi fermented *Camellia sinensis* after 30 min on glass beads revealed that semi fermented *Camellia sinensis* has more biofilm inhibitory effect (Fig. 4) that confirms the results of MICs by Well diffusion plates. Complementary test on the slide surface observed under phase contrast microscope showed significant reduction in attachment of these bacteria together (Fig. 5) while 30 min after treatment of bacteria with 1 mg mL⁻¹ of semi fermented and 1.5 mg mL⁻¹ of non fermented *Camellia sinensis* extracts the bacteria were not able to attach each other and have poor colonization for biofilm formation. These bacteria didn't have successful colonization and were more vulnerable to antibacterial agents.

The effectiveness of antimicrobial agents decreases with increasing age of the biofilm. Intact biofilms are also more resistant than disrupted communities (Wilson, 1996). The reasons for such differences are related to the community's properties and the bacterial cell changes that they induce.

Significant reduction in adherence of *S. mutans*, *S. mitis* and *S. sanguis* to glass surface, represent decrease in the cariogenic activity caused by adhesion of these bacteria specially *S. mutans* because this ability is considered as an essential step in the initiation and development of dental caries. Elvin-Lewis and Steelman (1968) claimed to have noted statistically improved dental health in children who drank at least one cup of tea daily compared to the dental health of those whose intake was less than 3 cups per week (Kubo *et al.*, 1993).

Although antimicrobial activity was expected based on the presence of some common components with tea like volatile components that exhibit this property and

possible anti-cariogenic activity related to the reduction of oral streptococci's adherence. These data are very promising considering that adherence is one of the main virulence factor of this species. Other studies including anti-adhesive activity on dental enamel and dentine and also *in vivo* studies are essentially necessary to highlight the clinical applications of these findings.

In conclusion, semi fermented *Camellia sinensis* inhibits the adherence of *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus sanguis in vitro* much better than non fermented *Camellia sinensis* extract at the same concentrations employed in this study due to rich content of volatile components exist in semi fermented extracts that can be very useful for controlling dental caries.

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