Original Article

In vitro antibacterial activity of *Camellia sinensis* extract against cariogenic microorganisms

Abstract

Context: Dental caries, a ubiquitous multifactorial infectious disease, is primarily caused by microorganisms like *Streptococcus mutans* and *Lactobacillus acidophilus*. Use of antimicrobials is an important strategy to curb cariogenic microorganisms.

Aim: The aim was to evaluate the *in vitro* antimicrobial activity of *C. sinensis* extract on *S. mutans* and *L. acidophilus.*

Study Setting and Design: Experimental design, *in vitro* study, lab setting.

Materials and Methods: Aqueous, acetone and ethanolic extracts of *C. sinensis* were subjected to antioxidant analysis. The ethanolic extract was used for assessment of antimicrobial properties. Ethanolic green tea extract at ten different concentrations and 0.2% chlorhexidine was used. Microbiological investigations were carried out to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and zone of Inhibition of the test and control agents against *S. mutans* and *L. acidophilus.*

Statistical Analysis: Kruskall–Wallis and Mann–Whitney U-test.

Results: MIC of green tea extract on *S. mutans* and *L. acidophilus* was found to be 0.2% and 0.3% respectively, MBC was found to be 0.8% and 0.9%, respectively. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *S. mutans* were 18.33 mm and 14.67 mm, respectively. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *L. acidophilus* were 12.67 mm and 7.33 mm, respectively.

Conclusion: Green tea has antibacterial activity against predominant cariogenic bacteria namely *S. mutans* and *L. acidophilus*.

Key words:
Camellia sinensis, dental caries, green tea, phytochemical analysis

Introduction

Dental caries is one of the most common oral infectious disease in man.[1,2] Oral microorganisms play a significant role in the etiology of dental caries. Over 400 species of microbes inhabit as commensals in the oral cavity of a healthy adult.[3] An aberration to this ecology due to dietary habits, improper oral hygiene or systemic factors lead to an increase in cariogenic microorganisms.[4]

Cariogenic microorganisms like *Streptococcus mutans and Lactobacillus acidophilus* encourage the accumulation and adherence of plaque biofilm by metabolizing sucrose into sticky glucan. Besides, the microorganisms in dental plaque degrade the dietary carbohydrates producing lactic acid leading to localized demineralization and the eventual formation of dental caries.[5,6] Although mechanical plaque control methods like brushing and flossing help to limit the growth of oral microbes, removal of plaque from inaccessible areas of the oral cavity remains a challenge. Hence, the use of the antimicrobial agent is warranted to limit the growth of cariogenic microorganisms and prevent dental caries.[3] Chlorhexidine, triclosan, cetyl pyridinium chloride, essential oils, fluoride based solution are some of the antimicrobial agents tested against oral microbes.[7-9] Chlorhexidine is the gold standard chemical plaque control agent. Its ability to bind with soft and hard tissues in the oral cavity enables it

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to act for a long period after application. However, brown discoloration of dentition and restorative material, dorsum of the tongue, taste perturbation, oral mucosal ulceration, unilateral/bilateral parotid swelling and enhanced supra gingival calculus formation, have been reported as side effects of long term chlorhexidine use.[10,11]

Drug resistance and side effects encountered with the use of synthetic drugs has led to the surge for novel and safe alternatives.[4] Since ancient times, plants have proved to be an archetypal source of medicine. *Camellia sinensis* is a shrub of the Theaceae family usually clipped to a height of 2–5 feet in cultivation, grown in semi-tropical environment[12]. Leaves are usually handpicked and based on the processing of the leaves three different types of tea are produced, namely Green tea (non-fermented), Oolong tea (semi-fermented) and Black tea (fermented).[13] Green tea being the non-fermented type possess more Catechin than the other two types.[4] Green tea has antioxidant, antiviral and antitumoral properties.[4,5] Despite abundant literature on the general health benefits of drinking green tea exists, its effect on cariogenic microbes is limited. With this background, the present study was carried out to determine the *in vitro* antibacterial effect of *C. sinensis* extract on *S. mutans* and *L. acidophilus*.

Materials and Methods

Materials, chemicals, and reagents

Green tea leaves (Gtee Botanicals, Chennai). Acetone; ethanol; n-butanol; methanol (Fischer Scientific, Mumbai), Folin and Ciocalteaus reagent (Loba Chemie, Mumbai), Muller Hinton media (Hi media, Mumbai), Paper discs (Hi media, Mumbai), 2-Diphenyl-picrylhydrazyl (DPPH); Butylated Hydroxy Toluene (BHT) (Sigma Aldrich, USA), Ultraviolet (UV) double beam spectra scan (Chemito, India).

Preparation of green tea extracts

This study was conducted after obtaining clearance from Institutional review board of Ragas Dental College, Chennai. *C. sinensis* leaves were procured from Gtee Botanical Extract Private Limited, Chennai. The physical and botanical characteristics of the leaves were authenticated by an expert botanist. 15 g of dried green tea leaves were mixed with 150 ml of acetone or ethanol (75%) or aqueous for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature and filtered using Whatman No. 1 paper. The filtered solution was evaporated under vacuum at 40°C to a constant weight and then dissolved in respective solvents.[14,15]

Phytochemical screening of green leaf extracts

Phytochemical screening of green tea extracts with acetone, ethanol and aqueous as solvents was done using standard methods.[16-18] The presence of major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins and steroids were analyzed.

Qualitative analysis of antioxidant activity of green tea

The qualitative antioxidant activity of the green tea extracts using all the three solvents were determined using DPPH.[19] 50 µl of extracts of green tea was taken in the microtiter plate. 100 µl of 0.1% methanolic DPPH was added over the samples and incubated for 30 min in dark condition. The samples were then observed for discoloration. The antioxidant positive samples were subjected for further quantitative analysis.

Quantitative analysis of free radical scavenging activity of green tea

The antioxidant activities were determined using DPPH (Sigma-Aldrich, USA) as a free radical.[20] A volume of 100 µl of green tea extract was mixed with 2.7 ml of methanol and 200 µl of 0.1% of methanolic DPPH and incubated for 30 min. Subsequently, at every 5 min interval, the absorption maxima of the solution were measured using a UV double beam spectra scan (Chemito, India) at 517 nm. Absorption of the blank sample containing the earlier mentioned amount of methanol and DPPH solution was prepared and measured as a control. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) of BHT. Free radical scavenging activity was calculated by the following formula:

$$\text{Inhibition} = \frac{[(\text{Absorbance of control (Ac517)} - \text{Absorbance of sample (As517)})}{(\text{Absorbance control (Ac517)})]} \times 100$$

Antioxidant activity of green tea extract using all the three solvents was determined. Heretofore, the extract that possessed greater antioxidant activity was used for further procedures.

Estimation of total phenol, flavonoid, tannin contents in Green tea

Total phenolic content in the ethanolic green leaf extracts of *C. sinensis* was determined by the Folin-Ciocalteau colorimetric method.[21] 0.5 ml of green tea extract was added to 0.1 ml of Folin-Ciocalteau reagent (0.5 N) and mixed thoroughly. Later 2.5 ml of Sodium carbonate was added, and the mixture was allowed to stand for 30 min in dark at room temperature. Gallic acid was used as a standard for the calibration curve and plotted at 0.2, 0.4, 0.6, 0.8 and 0.1 mg/ml, respectively. The absorbance was measured at 765 nm in a UV-visible spectrophotometer. Total phenolic content was expressed as milligram of gallic acid equivalents (GAE)/100 g of samples.

Total flavonoid content in the ethanolic green tea extract was determined by the aluminum chloride colorimetric method.[22] 0.5 ml of green tea extract, 0.1 ml aluminum chloride (10%), 0.1 ml of potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Quercetin was used as standard for the calibration curve, and plotted at 0.08, 0.16, 0.24, 0.32 and 0.40 mg/ml respectively. Absorbance was recorded at 415 nm in a UV-visible spectrophotometer. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent per gram of sample.

Tannin content of green tea leaf extract was estimated using Folin-Ciocalteau's reagent.[23] 1 ml of green tea extract was mixed with Folin-Ciocalteau's reagent (0.5 ml), then 1 ml of sodium carbonate solution and 8 ml of distilled water was added and allowed to stand for 30 min at room temperature. Tannin acid was used as standard for the calibration curve,

and plotted at 0.08, 0.16, 0.24, 0.32 and 0.40 mg/ml respectively. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using UV-visible spectrophotometer. The tannin content was expressed as mg tannic acid equivalent (TAE)/gram of the sample.

Antibacterial activity of green leaf extracts

Extraction of active compound

The fine powder of the green tea leaf was macerated with 75% of ethanol and evaporated. The residue was mixed with n-butanol and water (2:1). The upper layer of n-butanol and lower layer of water were separated and evaporated under vacuum. The upper layer of n-butanol residues was washed with petroleum ether to remove fatty components and then extracted with ethanol.[24]

Culturing of freeze dried Streptococcus mutans

The freeze dried *S. mutans* strains (MTCC 890) obtained from IMTECH, Chandigarh were made viable by transferring to Brain heart Infusion Broth (Hi media Laboratory Pvt. Ltd, Mumbai, India) and incubating at 37°C for 24 h. *L. acidophilus* strains were obtained from those isolated and maintained by Poonga Biotech Research Center, Chennai.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of green tea against both the bacteria was assessed by serial dilution method. Ten different concentrations of the green tea ethanolic extract (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7% 0.8%, 0.9%, 1.0%) were incorporated into nutrient broth in different test tubes. In each test tube, 5 ml of green tea extract was added to 4.9 ml of nutrient broth and 0.1 ml of bacterial culture. A control tube containing the growth medium and the bacteria was set-up. The mixtures were incubated at 37°C for 24 h and analyzed for turbidity. The minimum concentration of green tea extract that will inhibit the growth of the microorganism was determined as MIC.

Minimum bactericidal concentration

Minimum bactericidal concentration (MBC) was determined from MIC range using Spread plate method. Mueller Hinton agar in Petri dishes were sub-cultured from tubes without growth and incubated at 37°C for 24 h. The petri dishes were observed macroscopically. The highest dilution that yielded no bacterial colony on a solid medium was taken as MBC.

Zone of inhibition

Antibacterial activity was measured using the standard method of diffusion disc plates on agar.[25] 0.1 ml of each culture of bacteria was spread on agar plate surfaces. For antibacterial assay, all bacterial strains were grown in Muller Hinton Broth Medium (Hi media) for 24 h at 37°C and plated on Muller Hinton Agar (Hi media). Paper disc (6 mm in diameter) were placed on the agar medium to load 10 µl containing 100 µg, 20 µl containing 200 µg and 30 µl containing 300 µg of ethanolic extract of green tea. Inhibition diameters were measured after incubation for 24–48 h at 37°C. Control disks were prepared by infusing with 0.2% chlorhexidine. The plates were incubated at 37°C for 48 h. After 48 h, antibacterial activity of the extract against both the test bacteria was observed by growth free zone of inhibition near the respective disc.[25]

Statistical analysis

The data obtained was subjected to statistical analysis. The mean zone of inhibition was compared using Kruskall–Wallis followed by Mann–Whitney U test for pairwise comparison. Data analysis was performed using Statistical package for Social Sciences (SPSS) (SPSS Version 19, IBM Corp, New York). For all comparison $P < 0.05$ was considered as significant.

Results

Phytochemical analysis revealed that tannins, phenols, alkaloids, flavonoids, quinones, cardiac glycosides, terphenoids were present in *C. sinensis* extract irrespective of the solvent used [Table 1]. Glycosides were absent in all the three extracts. Qualitative analysis showed that ethanolic and aqueous extracts had stronger antioxidant property which was substantiated by quantitative analysis presenting 93.90% antioxidant activity for ethanolic extract. Whereas aqueous and acetone extracts had 89.02% and 79.26%, respectively [Figure 1]. Henceforth, all analysis were carriedout only with ethanolic extract of green tea. The total phenol content was found to be 96 mg GAE/g. The total flavonoid and tannin content was found to be 7.5 mg QE/g and 243.75 mg TAE/g, respectively [Table 2].

Table 1: Phytochemical screening of green tea extracts

Phytochemicals	Green tea extracts		
	Acetone	Ethanol	Aqueous
Tannins	+ +	+ +	+ +
Saponins	-	+ +	+
Flavonoids	+	+	+
Quinones	+ +	+	+
Glycosides	-	-	-
Cardiac glycosides	+ +	+ +	+
Terpenoids	+ +	+	+
Phenol	+ +	+ +	+
Coumarins	+	+	-
Steroids	+	+	+
Alkaloids	+ +	+ +	+
Antho cyanin	-	-	-
Beta cyanin	-	-	-

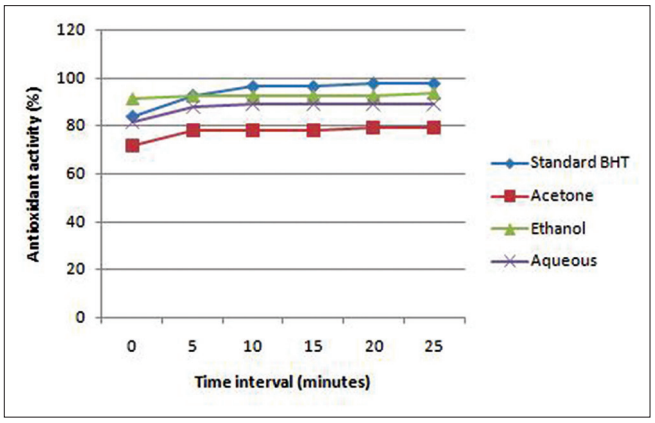

Figure 1: Antioxidant activity of acetone, ethanol and aqueous extracts of green tea

Camellia sinensis extract exhibited antibacterial effect on *S. mutans* and *L. acidophilus*. The MIC for *S. mutans* and *L. acidophilus* was 0.2% and 0.3% respectively [Table 3]. The MBC for *S. mutans* and *L. acidophilus* is 0.8% and 0.9% respectively. Zone of inhibition was assessed in triplets [Figure 2]. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *S. mutans* were 18.33 mm and 14.67 mm respectively, and the difference was found to be statistically significant. The mean zone of inhibition for 30 µl containing 300 µg of ethanolic extract of green tea and control against *L. acidophilus* were 12.66 mm and 7.67 mm respectively and this difference was found to be significant [Table 4].

Discussion

"Better to be deprived of food for 3 days, than tea for one" an ancient Chinese proverb elucidates the significance of tea. Although native of China, tea is widely used in the world for over 2000 years and is the second largely consumed drink in the world after water.[4,26] In recent days, there is an increased recognition on the versatile health benefits of green tea. Hence, this study was done to assess the anticariogenic, oral health benefits of green tea.

Microorganisms like *S. mutans, Lactobacillus* species, *Actinomyces naeslundii*, *Actinomyces viscosus*, *Rothia dentocariosa*, *Propionibacterium, Prevotella*, *Veillonella*, *Bifidobacterium, Scardovia* and *Enterococcus faecalis* are associated with the etiology of dental caries.[27] Despite the complexity of oral flora, there is convincing evidence that *S. mutans* and *L. acidophilus*, being acidogenic and aciduric are the main culprit microorganisms in the etiology of dental caries.[28] Hence in this study the antimicrobial activity of these two microorganisms were tested.

Qualitative analysis revealed that various phytochemicals were present. Tannins, phenols, alkaloids, flavonoids were present in *C. sinensis* extract irrespective of the solvent used. The flavonoids possess anti glycosyl activity and can inhibit adherence of microbes. Tannins can inhibit both glucosyl transferace (GFT) activity and bacterial growth by their strong iron-binding capacity. Alkaloids interfere with the division of cells thus inhibiting their growth.

In this study tannin, phenol and flavonoids content was found to be 243.75 mg/g, 96 mg/g and 7.5 mg/g, respectively. In a study done by Subramaniam *et al.* in 2013 tannin, phenol and flavonoid content of green tea was found to be 0.308 mg/g, 0.0105 mg/g and 350 mg/g respectively.[4] This variation in the chemical constituents in green tea may be attributed to the variation in geographic location of plant specimen like soil and climate.

Table 2: Total phenol, flavanoid and tannin content in green tea extracts

Phytochemical constituent	Total content present in green tea extract
Total phenolic content (mg GAE/g)	96
Total Flavonoid content (mg QE/g)	7.5
Total Tannin content (mg TAE/g)	243.75

GAE: Gallic acid equivalents, TAE: Tannic acid equivalent

Table 3: Minimum inhibitory concentration of *streptococcus mutans* and *lactobacillus acidophilus*

Minimum inhibitory concentration (%)	Absorbance values of *streptococcus mutans* at 720 nm	Absorbance values of *lactobacillus acidophilus* at 720 nm
0.1	0.25	0.26
0.2	0.20	0.22
0.3	0.20	0.21
0.4	0.20	0.21
0.5	0.20	0.21
0.6	0.20	0.21
0.7	0.20	0.21
0.8	0.20	0.21
0.9	0.20	0.21
1.0	0.20	0.21

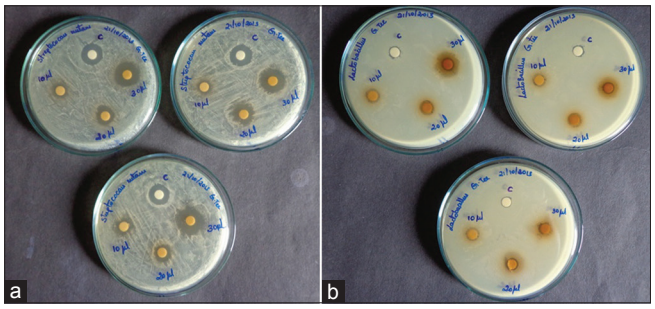

Figure 2: (a) Zone of inhibition of *Streptococcus mutans* against green tea extract at three different concentrations. (b) Zone of inhibition of *S. mutans* and *Lactobacillus acidophilus* against green tea extract

Table 4: Zone of Inhibition of *Streptococcus mutans* and *Lactobacillus acidophilus* against green tea extract

Microorganism tested	Mean (SD)				*P*	Intergroup comparison *P*
	Group I (100 µg) green tea extract	Group I I (200 µg) green tea extract	Group III (300 µg) green tea extract	Control Chlorhexidine (0.2%)		
Streptococcus mutans	10.00 (0.01)	12.66 (0.58)	18.33 (0.58)	14.67 (0.58)	0.01	Group III vs Group IV-0.03
Lactobacillus acidophilus	8.33 (0.58)	10.00 (0.01)	12.67 (0.58)	7.33 (0.58)	0.01	Group I, II, III vs Group IV-0.03

SD: Standard deviation

Among the 4000 bioactive compounds present in Green tea, the active constituents are polyphenols.[4] The major catechins present in green tea polyphenols are epicatechin EG, epigallocatechin (EGC), EGC gallate (EGCG), EGCG.[5] EGCG can cause cell membrane disruption and prevent DNA super coiling eventually leading to bacterial destruction. EGC interacts with proteins and distorts their tertiary structure. Since cariogenic bacteria have the ability to produce lactic acid that erode the tooth surface and can encourage the plaque adherence by producing sticky dextran from sucrose. GFT is a key enzyme, which metabolizes dietary sucrose.[12]

The study done by Tahir *et al*. in 2011 obtained MIC and MBC value of 0.7%, whereas in this study MIC of 0.2% and MBC of 0.3% was obtained.[2] These reduced values can be attributed to the higher tannin content in the sample studied. The ethanolic extract had greater antioxidant activity compared to acetone and aqueous extracts. This finding is in agreement with that of Subramaniam *et al*., 2013 in which ethanolic extract had greater antioxidant activity than aqueous extract.[4]

The inhibitory zones increased with increasing volumes of green tea ethanol extracts. It is remarkable to note that greater zone of inhibition was obtained for 300 µg/ml of green tea than Chlorhexidine against both *S. mutans* and *L. acidophilus*. As with any *in vitro* studies this study is not without limitations. This study has been performed in planktonic cultures of *S. mutans* and *L. acidophilus*. *In vivo* studies in this area are further required as limited data are available regarding their biofilm counterpart. Hence, further studies are required for understanding antimicrobial effect of green tea extract in biofilm.

Conclusion

Green tea has antibacterial activity against predominant cariogenic bacteria namely *S. mutans* and *L. acidophilus*.

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