

Inhibitory Activity of Green Tea (*Camellia sinensis*) Extract on Some Clinically Isolated Cariogenic and Periodontopathic Bacteria

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Key Words

Green tea · *Streptococcus mutans* · Periodontopathic · Minimal inhibitory concentrations

Abstract

Objective: To determine the in vitro inhibitory activity of green tea extract on some clinically isolated cariogenic and periodontopathic bacteria. **Materials and Methods:** Twenty strains of each of *Streptococcus mutans*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* were isolated from carious teeth and periodontal pockets of patients with dental caries and periodontal diseases. Green tea extract was prepared by aqueous extraction method and diluted from 50 to 1.56 mg/ml. Standard techniques of agar disk diffusion and broth microdilution assays were applied for qualitative and quantitative determinations of antibacterial activity of green tea extract on each isolates. **Results:** All clinical isolates of *S. mutans* (100%) were sensitive to green tea extract at concentrations 6.25, 12.5, 25, and 50 mg/ml producing inhibition zones ranging from 10 to 38 mm. All periodontopathic isolates (*A. actinomycetemcomitans*, n = 20, *P. intermedia*, n = 20, and *P. gingivalis*, n = 20) (100%) tested were sensitive to 12.5, 25, and 50 mg/ml of this extract. The minimal inhibitory concentration

of green tea extract for *S. mutans* was 3.28 ± 0.7 mg/ml and for *A. actinomycetemcomitans* 6.25, for *P. gingivalis* and *P. intermedia* 12.5 mg/ml. **Conclusions:** Our findings showed that green tea extract exhibited strong antibacterial activity on *S. mutans*, *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* and therefore may be used in mouthwashes or dentifrices for prevention of dental caries and periodontal diseases.

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Introduction

Green tea is one of the most popular beverages consumed worldwide. Moreover, during the last two decades it has received much attention in regard to its beneficial effects on various human health problems [1]. Tea prepared from *Camellia sinensis* is of three types: nonfermented green tea that is panfried or steamed and dried to inactivate its enzymes, fermented black tea and semifermented oolong tea. Green tea with active chemical ingredients possesses diverse pharmacological properties which are linked to lower incidence of some pathological conditions including oral cancer, dental caries, stroke, cardiovascular diseases and obesity [1–3].

Moreover, various reports on antimicrobial, antifungal, antioxidant, and cholesterol lowering activities of green tea and its constituents are documented [2–7]. The health-promoting effects of green tea are mainly attributed to its polyphenol contents commonly referred to as catechins. There are four main types of catechins: epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin-3-gallate and epicatechin [3]. The polyphenol contents of green tea have been reported to inhibit varieties of pathogenic bacterial growth such as *Helicobacter pylori*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sobrinus*, *Salmonella typhi*, *Shigella dysentery*, *Shigella flexneri* and *Vibrio cholera* [3, 5–9]. Green tea polyphenols were also found effective against human immunodeficiency virus, hepatitis, and influenza viruses [10, 11]. Dental caries and periodontal diseases are the two most prevalent plaques associated with oral infectious diseases produced by endogenous oral flora. *S. mutans* and *S. sobrinus* are known as the main etiological agents of dental caries. These cariogenic bacteria adhere to the tooth surface and produce a sticky glycocalyx film composed of glucan resulting from the action of glucosyltransferase on dietary sucrose. Accumulation of bacteria causes dental plaque formation within which there is continuing acid production by the bacterial plaque. Accumulation of high acid concentration in the plaque causes demineralization of enamel which consequently leads to caries formation. Periodontitis is a chronic slowly progressive polymicrobial infectious disease which affects the entire tooth-supporting tissues. This infection is characterized by destruction of alveolar bone, periodontal ligaments and gingival pocket formation which consequently leads to tooth loss. Periodontitis is known to be caused by subgingival plaque bacteria including *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Tannerella forsythus* and *Fusobacterium* species. These bacteria are frequently isolated from gingival pocket and subgingival plaques of patients with periodontitis. In the present study we investigated reporting the inhibitory activity of green tea extract (GTE) on some clinically isolated cariogenic and periodontopathic bacteria.

Materials and Methods

Isolation of *S. mutans* from Carious Teeth

S. mutans were isolated from carious teeth as described previously [12]. Briefly, the extracted carious teeth were incubated in 10 ml Todd-Hewitt broth (Merck, Germany) at 37°C,

5% CO₂ for 48 h. A Mitis-Salivarius-bacitracin agar was then subcultured from Todd-Hewitt broth and incubated at 37°C, 5% CO₂ for 72 h. *S. mutans* were then identified by biochemical tests [12]. Pure cultures of each clinical isolate of *S. mutans* were cultivated on Mitis-Salivarius-bacitracin agar and kept at 4°C until used.

Isolation of Periodontopathic Bacteria

Patients with either aggressive or localized aggressive periodontitis were examined and sampled for isolation of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* [13]. Subgingival pocket samples were taken from the deepest part of periodontal pockets (probing depth ≥6 mm) by insertion of a sterile paper point (Iso 35, Bocht, Offenburg, Germany). Each sample was inoculated into 5 ml of Trypticase soy broth containing hemin and menadione (Becton Dickinson Microbiology System) and kept under anaerobic conditions at 37°C, 5% CO₂ for 48 h. Bacteria from Trypticase soy broth were then subcultured on Trypticase soy-blood agar (TSBA) plates and kept under anaerobic conditions, 5% CO₂, 37°C for 72 h. The periodontopathic bacteria were isolated and characterized as reported elsewhere [13]. Pure cultures of each isolate were prepared on TSBA and kept at 4°C until used.

In the present study 20 clinical strains of each of *S. mutans*, *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* isolated from carious teeth and periodontal pockets of patients with periodontitis were used for GTE antibacterial activity by standard methods.

Preparations of Green Tea Extract

Green tea used in this study had been grown in Lahijan tea farm, in the northern part of Iran and has been purchased from a local store. GTE was prepared according to Yam et al. [5] with minor modifications. Briefly, 200 g of dried crushed green tea leaves were extracted into 500 ml boiling distilled water for 2 h. Insoluble particles were then removed by high speed centrifugation and filtration. The filtrate was reduced to 120 ml in a rotary evaporator and extracted 5 times with 180 ml of ethyl acetate. The organic phases were combined and concentrated to about 25 ml in a rotary evaporator and 25 ml of distilled water were added and the remaining ethyl acetate and aqueous phase was freeze-dried. Five grams of the dried precipitate was dissolved in 100 ml of phosphate-buffered saline (pH = 7.4) and used as stock solution of GTE (50 mg/ml). Twofold dilutions from the stock solution, i.e., 25, 12.5, 6.25, 3.12, and 1.56 mg/ml were prepared and used for antibacterial assays.

Antibacterial Assays

Standard agar disk diffusion (ADD) and broth microdilution methods were used to determine the inhibitory activity of GTE both qualitatively and quantitatively.

Agar Disk Diffusion

The cariogenic and periodontopathic bacteria isolates were subjected to ADD susceptibility testing using various dilutions of GTE on semisolid media [12, 13]. Mitis-Salivarius-Bacitracin agar plates were used for *S. mutans* and TSBA plates for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. *S. mutans* ATCC 25175, *A. actinomycetemcomitans* ATCC 29523 and *P. gingivalis* ATCC33277 were used as standard control isolates. These strains

were maintained anaerobically on TSBA supplemented with 10% defibrinated horse blood and hemin (5 µg/ml; Wako Pure Chemical Industries, Osaka, Japan). A pure bacterial cell suspension of each clinical isolate was prepared in 5 ml of Todd-Hewitt broth (*S. mutans*) or 5 ml Trypticase soy broth (*A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*); the suspension turbidity was adjusted to 1.5×10^8 colony-forming units (CFU)/ml equivalent to McFarland standard No. 0.5. A 100-µl sample of this suspension was seeded onto semisolid appropriate media. A 6-mm-diameter sterile Whatman filter paper No. 5 (round filter Machery-Nagel, Doren, Germany) was impregnated with 50 µl of various concentrations (50, 25, 12.5, 6.25, 3.12, 1.56 mg/ml) of GTE, placed on the above agar culture media and incubated at 37°C under anaerobic condition for 72 h. The diameter of the zone of growth inhibition around the disk was measured in millimeters, mean and standard deviation (SD) were calculated and recorded. Disks containing GTE which did not produce inhibition zones were considered as negative results. Sterile filter paper disks soaked in 50 µl of phosphate-buffered saline, pH = 7.4, and antibiotic disks of vancomycin (30 µg) and amikacin (30 µg) were also used as controls.

Broth Microdilution Method for Determination of Minimal Inhibitory Concentration

Broth microdilution methods were carried out in 96-well culture plates to determine the minimal inhibitory concentration (MIC) of GTE against cariogenic and periodontopathic isolates [13, 14]. Todd-Hewitt broth was used for *S. mutans* and Trypticase soy broth containing menadione and hemin was used for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. Bacterial cell suspensions of each of the clinical isolates were prepared in the above liquid media and their concentrations were adjusted to 10^7 CFU/ml. Twofold dilutions of GTE were prepared in the appropriate broth culture media from stock solution. Aliquots (200 µl) of each dilution of GTE were dispensed in 96-well culture plates. One hundred microliters of each bacterial suspension was added to each well and incubated under anaerobic conditions in 5% CO₂ at 37°C for 48 h. The absorbance was then measured at 595 nm. The highest dilution of GTE at which no growth (OD ≤ 0.05) was observed, was defined as MIC.

Results

The results of GTE antibacterial activity on the cariogenic and periodontopathic bacteria studied by ADD tests are given in table 1.

All isolates of *S. mutans* (100%) were sensitive to the extract at concentrations of 6.25, 12.5, 25, and 50 mg/ml and exhibited growth inhibition zones ranging from 10 to 38 mm. At 3.12 mg/ml, 18/20 (90%) of *S. mutans* isolates were sensitive, producing inhibition zones of 6–10 mm. *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were sensitive to GTE at concentrations of 12.5, 25, and 50 mg/ml and produced inhibition zones ranging from 10 to 30 mm in diameter (table 1). At the con-

Table 1. Inhibitory activity of various concentrations of GTE on some clinically isolated cariogenic and periodontopathic bacteria by ADD test

	<i>S. mutans</i> (n = 20)	<i>A. actinomycetemcomitans</i> (n = 20)	<i>P. gingivalis</i> (n = 20)	<i>P. intermedia</i> (n = 20)
GTE concentration, mg/ml				
1.56	R	R	R	R
3.12	7.5±2.76	R	R	R
6.25	12.3±1.62	7.35±3.3	7.25±3.22	R
12.5	22±1.52	12.5±1.8	11.65±1.53	11.85±1.53
25	26.9±1.51	17.35±1.72	16.8±1.6	16.85±1.53
50	36.3±1.08	27.25±1.65	26.65±1.56	27.05±1.6
Vancomycin	17 ^a	0	0	0
Amikacin	0	16 ^a	16 ^a	16 ^a
PBS	0	0	0	0

Values are mean diameters of inhibition zones in millimeters ± SD. PBS = Phosphate-buffered saline, pH = 7.4; R = resistant; n = number of isolates. Vancomycin (30 µg) and amikacin (30 µg) were used as reference antibacterial compounds.

^a Mean diameter of zone of inhibition of 3 experiments.

Table 2. MICs of GTE (mg/ml) on some clinically isolated cariogenic and periodontopathic bacteria by broth microdilution method

	<i>S. mutans</i> (n = 20)	<i>A. actinomycetemcomitans</i> (n = 20)	<i>P. gingivalis</i> (n = 20)	<i>P. intermedia</i> (n = 20)
GTE	3.28±0.7	6.25±0	12.5±0	12.5±0
Vancomycin	30 ^a	0	0	0
Amikacin	0	30 ^a	30 ^a	30 ^a

Values are mean MICs of GTE ± SD.

^a MICs of vancomycin and amikacin are the mean of 3 experiments in micrograms per milliliter.

centration of 6.25 mg/ml of GTE, 75% (n = 15) of *P. gingivalis*, 70% (n = 14) of *A. actinomycetemcomitans*, and 65% (n = 13) of *P. intermedia* were sensitive. None of the bacterial isolates were sensitive to 1.56 mg/ml or less. The MIC results of GTE on *S. mutans* and periodontopathic bacteria are shown in table 2. The MIC of GTE for *S. mutans* was 3.28 ± 0.699 and for *A. actinomycetemcomitans* was 6.25, for *P. gingivalis* and *P. intermedia* 12.5 mg/ml. The MICs of GTE on *S. mutans* (ATCC 25175), *A. actinomycetemcomitans* (ATCC 29523) and *P. gingivalis* (ATCC 33277) were 6.25, 12.5, and 12.5 mg/ml, respectively.

Discussion

The crude GTE exhibited strong inhibitory activity on *S. mutans*, *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. However, *S. mutans* was more susceptible than periodontopathic bacteria as crude GTE produced growth inhibition zones up to 36.3 mm by ADD test (table 1) and MIC was as low as 3.28 mg/ml (table 2). Ikigai et al. [15] reported statistically significantly lower MIC of EGCG for *S. aureus* (73 µg/ml) versus MIC for *Escherichia coli* (183 µg/ml). The same authors also found epicatechin to be much less active than EGCG with MICs of 573 and ≤1,104 µg/ml for *S. aureus* and *E. coli*, respectively. In our study, the MIC of crude GTE was higher for Gram-negative periodontopathic bacteria (*A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*) than for Gram-positive bacteria (*S. mutans*). The higher MIC values of crude GTE and catechins for Gram-negative than Gram-positive bacteria of this study confirmed those of previous studies. Our study also confirmed the greater susceptibility of Gram-positive bacteria, an effect that might be due to differences in cell wall structure and polysaccharide charges of the bacteria. Our data revealed strong inhibitory activity of crude GTE on clinically isolated periodontopathic bacteria as this extract produced growth inhibition zones ranging from 11.65 to 27.25 mm in ADD and MIC of 6.25–12.5 mg/ml by broth microdilution method. The inhibitory activity of GTE on periodontopathic bacteria is attributed to polyphenol catechins and particularly EGCG. It has been shown that adherence and colonization of *P. gingivalis* on the buccal epithelial cells was completely inhibited in the presence of EGCG at concentrations of 250–500 µg/ml while other polyphenolic compounds were not as effective as EGCG at this concentration [16]. The precise mechanism of action of EGCG remains unclear, however, several mechanisms have been proposed for the anticariogenic and antiperiodontopathic activities of tea polyphenol galloylated catechins. EGCG has been shown to cause irreversible membrane disruption in both Gram-positive and Gram-negative bacteria [15] and also to inhibit bacterial DNA gyrase preventing DNA supercoiling and leading to bacterial cell death [17]. EGCG neutralizes toxic end metabolites such as collagenase, protein tyrosine phosphatase, alkaline phosphatase, and gingipains of periodontopathic bacteria, which causes destruction of gingival tissues and progression of periodontitis [18, 19]. Furthermore, EGCG has been shown to inhibit proliferation of *S. mutans*, interfere with the bacterial adhesion

process to the enamel and also suppress glucosyltransferase and amylase activities, which consequently leads to reduced acid production in the dental plaques [16, 20, 21].

Effective prevention of dental caries and periodontal disease could be achieved by proper and regular toothbrushing, flossing and rinsing with mouthwashes containing antibacterial agents such as chlorhexidine, sodium hypochlorite, cetylpyridinium chloride and amine fluoride. Moreover, chlorhexidine gluconate and sodium hypochlorite were shown to be cytotoxic to human periodontal ligament cells, inhibit protein synthesis, affect mitochondrial activity of these cells and consequently may have detrimental effects on vital tissues [22, 23]. Considering the potential disadvantages and side effects of these chemical agents, there is a need for more agents with marked antibacterial activity, greater sensitivity and less toxicity to be used as mouthwashes and irrigating agents. During the last decade much attention has been given to the antimicrobial activities of medicinal plants and their extracts to be consumed as useful alternatives to synthetic chemical agents [12, 13, 24, 25]. Among the medicinal plants, green tea is of particular interest and has been used therapeutically for a long time in various parts of the world [1–9, 16]. Most of the biological activities of green tea, particularly its antibacterial properties, have been associated with the polyphenol catechin fractions which constitute up to 30% of solid green tea leaves [1, 3]. EGCG is the most abundant of these catechins, comprising about 50% of the catechin pool [1]. It has been shown that catechin components of green tea and particularly EGCG, epigallocatechin and epicatechin-3-gallate, which are all catechin derivatives having a galloyl moiety linked by an ester linkage, constitute the most important antibacterial agents in GTE [1, 3, 16].

Several investigators have reported that catechins are inhibitory for *S. mutans* with MIC ranging from 50–1,000 µg/ml [16, 26]. Xu et al. [27] reported that EGCG inhibited growth of *S. mutans* at an MIC of 31.25 µg/ml and minimum bactericidal concentration of 62.5 µg/ml. On the other hand, Muroi and Kubo [28] found no significant antibacterial activity of EGCG against oral streptococci at 500 µg/ml. Sasaki et al. [26] reported that combination of EGCG, GCG and CG exhibited stronger antibacterial activity against *S. mutans* MT8148R whereas pure EGCG, GCG and CG products individually showed only a moderate or low level of antibacterial activity against *S. mutans*. Although EGCG is the most potent antibacterial catechin, it is reasonable to assume

that synergistic action may exist between the above-mentioned galloylated catechins and therefore consumption of crude GTE seems to be more useful than the pure catechins.

Green tea is safe for most of the people when used in moderate quantities. The most adverse effects of green tea administered orally are gastrointestinal upset and central nervous system stimulation from the caffeine content of the tea. There are several case reports of hepatotoxicity linked to GTE products in pill or beverage form, however, the mechanism of this symptom is not known [29]. Allergic reactions have been reported with topical green tea ointment, which may cause cervical and vaginal inflammation, irritation and vulvar burning [30].

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Conclusions

Data presented in this study revealed in vitro inhibitory activities of GTE on cariogenic and periodontopathic bacteria and concluded that they could be used in mouthwashes for dental caries and periodontal disease prevention.

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