

ANTIBACTERIAL EFFICACY OF 6% GREEN TEA EXTRACT AND 2% CHLORHEXIDINE AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM *IN VITRO*

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Received: 27 August 2018, Revised and Accepted: 07 February 2019

ABSTRACT

Objective: This study aimed to compare the antibacterial efficacy of green tea extract to chlorhexidine 2% against *Enterococcus faecalis* biofilm using real-time polymerase chain reaction (RT-PCR).

Methods: *E. faecalis* strain ATCC 29212 colonies were collected from overnight cultures grown on brain–heart infusion agar (BHIA) and resuspended at 10^8 CFU/ml according to a 0.5 McFarland standard. Aliquots of bacterial suspension (50 μ l) were then inoculated onto sterile nitrocellulose filter discs placed on BHIA and incubated aerobically at 37°C for 72 h. After incubation, the discs were removed, transferred into 10-ml phosphate-buffered saline (PBS) to loosen planktonic bacteria, and then incubated for 10 min at 37°C in 10 ml of 6% green tea extract, 2% chlorhexidine, or sterile PBS (control). Surviving *E. faecalis* cells were then quantified by RT-PCR.

Results: Green tea extract reduced bacterial survival compared to control but was not as effective as chlorhexidine 2%.

Conclusion: Green tea extract may help reduce oral *E. faecalis* biofilm. Moreover, specific antimicrobial compounds in green tea extract such as epigallocatechin-3-gallate should be tested as non-toxic alternatives to chlorhexidine.

Keywords: *Enterococcus faecalis*, Green tea extract, chlorhexidine 2%, Real-time polymerase chain reaction.

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INTRODUCTION

The success of root canal treatment is dependent on the effective elimination of microbes and irritants from the root canal system, which prevents reinfection and promotes healing. Bacteria remain in the root canal following mechanical cleaning due to the complexity of root canal anatomy, so an irrigation solution is also needed to clean and help remove residual bacteria. However, some bacterial species can survive even in prepared, irrigated, and obturated root canals, such as *Enterococcus faecalis*, which contributes to the persistence of periradicular lesions after root canal treatment. *E. faecalis* is resistant to several tested antibacterial agents, can invade and live in dentinal tubules, and can form biofilms that make it 1000 times more resistant to phagocytic agents, antibodies, and antimicrobials [1].

Many studies have examined the most effective way for removing *E. faecalis* from the root canal, and chlorhexidine has shown particularly high efficacy [2-5]. However, chlorhexidine is a synthetic chemical with known toxicity against certain cells, such as fibroblasts, polymorphonuclear leukocytes (PMNs), and other leukocytes, macrophages, and erythrocytes. Therefore, it would be advantageous to identify natural agents with strong antibacterial efficacy against *E. faecalis* but low toxicity against mammalian cells to enhance the biosafety of bacterial clearance during root canal treatment. For instance, green tea is known to have antioxidant, antimutagenic, anticarcinogenic, and antimicrobial effects [2,4]. While several studies have been conducted to test the antibacterial efficacy of green tea extract, none have directly compared its antibacterial effect to 2% chlorhexidine solution against *E. faecalis* bacteria in biofilm.

METHODS**Culture of *Enterococcus faecalis* ATCC 29212**

E. faecalis strain ATCC 29212 was smeared evenly onto brain–heart infusion agar (BHIA) and incubated for 24 h at 37°C, by which time

bacterial colonies were clearly visible. *E. faecalis* colonies were drawn into needles and transferred to test tubes containing 10-ml sterile saline, and the density was adjusted to 10^8 CFU/ml using a 0.5 McFarland standard solution.

Preparation of *Enterococcus faecalis* biofilm

A sterile cellulose nitrate filter membrane (pore size 0.2, 13 mm in diameter) was placed on top of BHIA, and 25 μ l of the standardized *E. faecalis* suspension was added using a micropipette. Membranes containing the bacterial suspension were incubated at 37°C for 72 h under aerobic conditions and then treated with phosphate-buffered saline (PBS) (control), chlorhexidine 2%, or 6% green tea extract as described below.

Preparation of green tea extract

Green tea extract was prepared from imperial green tea leaves obtained from the Tea and Kina Gambung Research Center (Bandung, West Java, Indonesia). Imperial tea is produced from tea leaf buds that have not yet bloomed and processed using a steaming system. Briefly, dried imperial green tea leaves were crushed and sieved to form a fine powder. A 250-g sample of tea powder was then fed into an extractor gourd with 2.5 l of distilled water. The mixture was placed over a heater to evaporate for 1 h at a temperature below 80 °C. The insoluble particles were removed by a Mesh 250 filter, and the filtrate was transferred to Erlenmeyer flasks. Initial filtrate volume (V1) was about 2200 ml. The extract content was tested (%E for V1) and then evaporated using a vacuum rotary evaporator at an operating temperature below 80°C until the final volume (V2) was about 180 ml and %E reached 45.50%. The green tea extract was diluted to 6% in sterile Aqua Bidest double-distilled water using the formula $C1.V1=C2.V2$ and filtered through 0.2- μ m membranes.

Treatment of *Enterococcus faecalis* ATCC 29212 biofilms

After incubation for 72 h, *E. faecalis* cultures growing in cellulose nitrate membranes on BHIA were transferred into Eppendorf tubes with 1 ml of

Table 1: Mean *Enterococcus faecalis* survival after treatment

95% IK treatment	n	Mean±SD	Lower limit	Upper limit
Green tea 6%	3	4.64×10 ⁷ ±0.12×10 ⁷	4.35×10 ⁷	4.93×10 ⁷
Chlorhexidine 2%	3	0.84×10 ⁴ ±1.29×10 ⁴	-2.36×10 ⁴	4.03×10 ⁴
Control (PBS)	3	16.6×10 ⁷ ±4.04×10 ⁷	6.57×10 ⁷	26.6×10 ⁷

SD: Standard deviation, PBS: Phosphate-buffered saline

Table 2: Statistical summary of pair-wise comparisons between individual treatment groups

Treatment	Green tea 6%	Chlorhexidine 2%	Control
Green tea 6%	-	0.05*	0.05*
Chlorhexidine 2%	0.05*	-	0.05*
Control	0.05*	0.05*	-

*p<0.05 significant by Mann-Whitney U-test

(PBS solution to release bacteria not firmly attached to the membrane (planktonic bacteria). Washed membranes were inserted into Eppendorf tubes containing either 1-ml PBS (control), 1 ml of 6% green tea extract, or 1 ml of 2% chlorhexidine solution (3 tubes and membranes per treatment group) and incubated for 10 min at 37°C in an anaerobic incubator.

Isolation of *Enterococcus faecalis* ATCC 29212 from biofilm following treatment

All membranes exposed to the test solutions were washed three times with 1-ml PBS, each time in a new Eppendorf tube, to neutralize and stop antibacterial activity. The third Eppendorf tube with the membrane was then vortexed for 2 min to obtain the bacterial suspension, and the membrane removed aseptically.

Photoconjugation of dead-cell DNA with propidium monoazide

After cell isolation and before DNA extraction and polymerase chain reaction (PCR) amplification, the bacterial suspension (100-µL samples in Eppendorf tubes) was treated with the photoreactive DNA dye PMA at a final concentration of 100 µM to prevent subsequent PCR amplification of DNA from dead cells. Briefly, tubes were incubated for 10 min at 4°C in a dark room, then placed horizontally on dry ice, and exposed to 600 Watt halogen rays at a distance of 20 cm for 20 min.

DNA extraction

A water bath incubator was prepared at 56°C and thermoblock units at 100°C. The bacterial sample was resuspended by vortexing for 10 s and centrifuged at 10000 rpm for 3 min. After centrifugation, the supernatant was removed by micropipette and discarded, leaving only the raw cell pellet. The pellet was then mixed with 200-µl InstaGene™ Matrix over a hot plate with magnetic stirring, incubated in a water bath at 56°C for 30 min, and mixed again by vortexing for 10 s. The tube was then inserted into the thermoblock at 100°C for 8 min. This procedure was repeated up to 3 times to solubilize DNA and precipitate cellular components that interfere with PCR.

The sample was then homogenized by vortexing for 10 s and centrifuged at 12,000 rpm for 3 min. The supernatant was transferred into a new microcentrifuge tube and stored at 4°C for 24 h. If deposit was still visible on the tube, the supernatant was transferred again into a new microcentrifuge tube and stored at -20°C for additional DNA purification.

Quantification of sample DNA concentration

A working solution was prepared by adding Quant-iT™ DNA-BR Buffer (199 µl × n where n=number of samples, in this case 9) into 15 ml centrifuge tubes pre-covered with aluminum foil. Then, 1 µl × 9 Quant-iT™ Reagent DNA-BR was added and mixed in using a vortex. This working solution (199 µl) was mixed with 1-µl sample DNA in a 0.5-ml microcentrifuge tube covered with aluminum foil, and the new mixture was vortexed for 2 s. Total DNA was then measured against a kit standard on a microplate reader at 502/523-nm excitation/emission.

Detection and Quantification of live *Enterococcus faecalis* ATCC DNA 29212

Primers EF Gro ES-F and EF Gro ES-R were diluted in TE buffer at 9:1, mixed by vortexing, and briefly centrifuged. A PCR mix was then prepared by multiplying each component volume by the number of samples to be examined using real-time PCR in 1.5-ml microcentrifuge tubes coated with aluminum foil. The final mixture contains 10-µl Power SYBR® Green PCR Master Mix I, 2-µl universal primer 357F, 2-µl universal primer 907R, and 2-µl nuclease-free water. Then, 16 µl was added to 4-µl DNA sample in MicroAMP™ Fast Reaction Tubes and mixed using a micropipette.

MicroAmp™ Fast Reaction Tubes (8 tubes/strips) were covered with the MicroAmp™ Optical 8-Cap Strip aseptically. A PCR well plate was then inserted into the Step One Real-Time PCR System (Applied Biosystems). The real-time PCR quantitative cycle was 95°C for 3 min (pre-activation), followed by 40 cycles of denaturation at 95°C for 15 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 30 s. After real-time PCR, the results could be read on the computer screen.

Statistical analyses

The numbers of surviving *E. faecalis* in samples from control, green tea, and chlorhexidine treatment groups were analyzed for normality and homogeneity of variance. If the data distribution was normal and the variance homogeneous, statistical analysis was performed using one-way ANOVA and *post hoc* least significant difference tests for pair-wise comparisons. If the data distribution was not normal or the data variance was not homogeneous, treatment groups were compared by the non-parametric Kruskal-Wallis test with *post hoc* Mann-Whitney U-tests for pair-wise comparisons.

RESULTS

Table 1 presents the mean (±standard deviation) number of surviving *E. faecalis* bacteria following control, green tea, or chlorhexidine treatment (n=3 samples/treatment group). The Kruskal-Wallis test indicated a significant difference among treatment groups (p=0.027), and *post hoc* pair-wise comparisons revealed significant differences between all groups (p≤0.05 by Mann-Whitney U-test). Thus, both green tea extract and chlorhexidine reduced *E. faecalis* numbers compared to control treatment. However, the final count was significantly lower following chlorhexidine treatment compared to green tea extract treatment. Table 2 presents the results of the Mann-Whitney U-test for each pair-wise comparison

DISCUSSION

Green tea extract was examined as a possible treatment to eliminate oral *E. faecalis* biofilm because it has demonstrated antimicrobial efficacy as well as additional health benefits without cellular toxicity. Indeed, 6% green tea extract did significantly reduce *E. faecalis* numbers in artificial biofilms compared to control treatment but not as effectively as 2% chlorhexidine [2,4]. Thus, green tea extract may help reduce *E. faecalis* biofilms although alone it is not as clinically effective as chlorhexidine. It is possible, however, that bioactive components in green tea extract may be high-potency natural agents for *E. faecalis* biofilm elimination.

The antibacterial activity of green tea is most strongly associated with a polyphenol catechin fraction containing about 30% of the natural epigallocatechin-3-gallate (EGCG), a compound that accounts for 50% of the total catechin content and can cause irreversible membrane

damage to Gram-positive bacteria. In addition, EGCG can also inhibit bacterial DNA gyrase enzymes by binding to ATP B subunits, preventing DNA supercoiling, and causing bacterial cell death.

The tea leaves used for extract preparation in this study were of the imperial type obtained from a research center for tea and quinine (PPTK Bandung). Imperial is a pekoe tea produced from young tea shoots, and it is known that younger tea shoots contain higher levels of polyphenols than mature leaves. According to PPTK Bandung, this type of pekoe tea has the highest polyphenol concentration (36.91%) and a catechin content of about 26.5%. In this study, the green tea solution was used at 6%, a concentration selected based on a study by Martina *et al.* reporting that concentrations of 3%–6% eliminate *E. faecalis* growth [3]. According to Martina *et al.*, 3% green tea extract had antibacterial activity comparable to 2% chlorhexidine [3]. In our preliminary experiments, however, *E. faecalis* growth was still observed in 3% green tea extract while growth of new *E. faecalis* bacteria was not detectable at 6%, comparable to results using 2% chlorhexidine. This difference in antimicrobial potency between our study and Martina *et al.* likely stems from differences in the natural polyphenol catechin content, which may be influenced by the geographic location of cultivation, growth conditions, processing, and extract preparation methods [3]. In this study, *E. faecalis* biofilm was exposed to each test material for 10 min, a duration chosen according to Kim *et al.*, who found that maximum attachment of antibacterial particles to the bacterial cell wall required more than 5 min and that 10 min was sufficient for antibacterial substances to adhere and cause substantial damage to the *E. faecalis* membrane [5].

Traditional microbiology methods used to quantify bacteria for toxicity analysis are time-consuming and non-specific. In contrast, PCR using specific primers allows for rapid and sensitive detection of individual species [6]. However, PCR techniques also have limitations, especially for assessing the effectiveness of an antimicrobial agent. Conventional PCR can only detect the presence or absence of bacteria but not the quantity, while real-time PCR of untreated bacteria cannot distinguish between living and dead cells because bacterial DNA is stable in the environment for long periods after cell death [7,8]. To overcome this problem, cells were first stained with propidium monoazide (PMA), which forms covalent bonds with DNA bases upon photoactivation, thereby inhibiting PCR amplification of DNA from dead cells but not living cells [9]. Further, PMA cannot penetrate the intact cell membrane and so will only react with the DNA from irreversibly damaged cells. By blocking PCR amplification of DNA from dead cells, it was possible to accurately determine the antimicrobial efficacies of green tea extract and chlorhexidine against *E. faecalis* in biofilms [10].

The antibacterial efficacy of green tea extract against *E. faecalis* cultured in artificial biofilm is in accord with Garg *et al.*, who found that green tea polyphenols have substantial antibacterial power against *E. faecalis* biofilm [9]. However, the green tea polyphenols tested in that study were extracted with dimethyl sulfoxide (DMSO) rather than water and so likely include unique compounds.

While green tea did reduce the number of surviving *E. faecalis*, total survival was still higher than after chlorhexidine 2% treatment. These results differ from those of Martina *et al.*, who reported that 3% green tea extract had antibacterial activity comparable to chlorhexidine 2% [3]. This difference may be explained by the different bacterial forms as planktonic bacteria are more easily eliminated than bacteria in biofilm. In biofilm, genetic and metabolic changes as well as the

formation of a thick extracellular polymeric substance block access of some antimicrobial agents, thereby enhancing resistance 500–1000-fold compared to planktonic cells [11]. As mentioned, differences in antimicrobial efficacy among studies are also related to the natural EGCG content of the extract. Thus, it is necessary to examine the antimicrobial efficacies of purified catechin compounds directly, particularly EGCG, as it is the most active and abundant component of catechin. Moreover, EGCG extract of green tea is commercially available in powder at 50%–98% and is already used as a natural ingredient for medicines, dietary supplements, beverages, and cosmetics due to its low toxicity.

CONCLUSION

Green tea extract solution had antibacterial efficacy against *E. faecalis* in biofilm. However, the efficacy of 6% green tea extract is lower than chlorhexidine 2% solution. Nonetheless, these results suggest that specific bioactive components of green tea extract, such as EGCG, are potentially effective non-toxic agents for the reduction of oral *E. faecalis* biofilm.

CONFLICTS OF INTERESTS

There are no conflicts of interest to declare.

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