

ANTIBACTERIAL EFFICACY OF GRAPE SEED EXTRACT CONTAINING 2.9% TANNIN AGAINST *ENTEROCOCCUS FAECALIS* BIOFILM

SHELVY SOETANTO, ENDANG SUPRASTIWI*, DINI ASRIANTI

Department of Conservative Dentistry, Faculty of Dentistry, Universitas Indonesia, Jakarta, 10430, Indonesia. Email: esuprastiwi@yahoo.co.id

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

ABSTRACT

Objective: The aim of this study was to observe the antibacterial efficacy of grape seed extract (GSE) against *Enterococcus faecalis* biofilm.

Methods: A biofilm of *E. faecalis* American Type Culture Collection (ATCC) 29212 strain was prepared using sterile cellulose nitrate filter membrane incubated on brain heart infusion agar at 37°C for 72 h under aerobic condition. Each membrane containing *E. faecalis* biofilm was added to three tubes of phosphate-buffered saline (control), three tubes of GSE, and three tubes of 2% chlorhexidine. The number of viable DNA cells was measured using real-time polymerase chain reaction. The data were statistically analyzed using non-parametric Kruskal-Wallis test and Mann-Whitney U-test.

Results: GSE had antibacterial efficacy against *E. faecalis* biofilm. The difference in the amount of *E. faecalis* DNA between all groups was statistically significant ($p=0.05$).

Conclusion: GSE has antibacterial efficacy against *E. faecalis* biofilm.

Keywords: Grape seed extract, Biofilm, *Enterococcus faecalis*.

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INTRODUCTION

The aim of root canal treatment is to prevent the progression of infection to the periapical tissue; however, the aim of periapical disease treatment is to create the ideal conditions for the disease to heal [1]. This goal can be achieved through instrumentation, disinfection, and obturation of the root canal. As the root canal has a complex anatomy, root canal preparation using mechanical instrumentation only partially eliminates the bacteria in the root canal [2]. It is expected that the combination of endodontic instrumentation and antibacterial agents can eliminate all infections on the periapical tissue [3].

Endodontic infections are polymicrobial. These microorganisms are usually opportunistic pathogens that are beneficial under normal conditions but can be pathogenic when they reach the pulp or periapical tissue [4]. The microorganisms involved in endodontic infections are bacteria, fungi, archaea, and viruses. *Candida albicans* is a common fungal species found in the oral cavity. According to a recent study, *C. albicans* was found in 21% of infected root canal cases. Archaea are prokaryotic microorganisms associated with various pathogenic infections. They can be detected in subgingival plaques with periodontal disease and infected root canals. The role of viruses in endodontic infections is still debatable. The viruses that may be detected in apical periodontitis are human cytomegalovirus and Epstein-Barr virus [5].

Kakehashi *et al.* (1965) stated that obligate anaerobic, facultative anaerobic, and obligate aerobic bacteria were the main cause of infection in endodontic cases [4]. Secondary infection is a major cause of endodontic treatment failure. It may occur when bacteria penetrate the root canal and adapt to the environment. Based on culture and molecular studies, *Enterococcus faecalis* is the most commonly found bacteria in endodontically treated teeth and can account for >90% of endodontic cases. This suggests that *E. faecalis* can survive in obturated root canals, even in environments that are not ideal for their growth [1]. *E. faecalis* is resistant to antibacterial agents, able to survive without nutrients for long periods of time, adaptable to environmental changes with high pH value, and protected by biofilms. A study by Rocas *et al.*

(2004) reported that *E. faecalis* is found 9 times more often in cases of endodontic failure than in cases of primary endodontic infections [6].

Sodium hypochlorite (NaOCl) is a widely used disinfectant in endodontic treatment, although the ability of NaOCl and the concentration used to eliminate *E. faecalis* is still debatable. However, Kandaswamy and Venkateshbabu (2010) stated that irrigation with 1.3% and 2.5% NaOCl was not effective in eliminating *E. faecalis* on infected dentin cylinders and that NaOCl effectively eliminated bacteria but was not effective against bacterial endotoxins [7].

Another disinfectant used to eliminate *E. faecalis* is chlorhexidine (CHX). CHX exerts its antibacterial action by binding to the cell walls of bacteria and causing leakage and lysis of bacterial cells. Ferraz *et al.* (2007) conducted an *in vitro* study to demonstrate the ability of CHX to eliminate *E. faecalis* and found that 2% CHX in both gel and solution form is effective in eliminating *E. faecalis* in the root canal and dentin tubules [8].

It is important that root canal disinfection material not cause irritation of the periapical tissue [2]. NaOCl and CHX are irritative to the periapical tissue [9,10]. Therefore, to improve biological safety, a natural antibacterial agent was developed. Grape seed extract (GSE) is used to treat various diseases due to its biological activities, such as antioxidant, anticancer, anti-inflammatory, and antibacterial activities [11].

GSE is derived from the seeds of *Vitis vinifera* and contains proanthocyanidins (PAs), which are phenolic compounds [12]. As an antibacterial agent, the PAs in GSE prevents oral cavity diseases such as periodontitis. PAs in grape seed ethanol extracts reportedly eliminate *Streptococcus mutans* [13]. In addition, GSE has an inhibitory effect on *E. faecalis* [14]. Angellina (2013) observed the effect of GSE solution in removing the smear layer in the root canal. The result showed that a 6.5% GSE removed the smear layer, particularly in the third apical of the root canal. The elimination of the smear layer is believed to be one of the ways to increase the effectiveness of root canal antibacterial agents. It may occur because antibacterial agents can better penetrate the root canal to avoid microleakage [15].

This objective of the present study is to observe the antibacterial effect of GSE solution on *E. faecalis* in biofilm using real-time polymerase chain reaction (RT-PCR), so it can be used as a safe antibacterial agent in endodontics in the future.

METHODS

This study was conducted at the Institut Pertanian Bogor from June to October 2014. The antibacterial efficacy of GSE solution with 2.9% tannin against *E. faecalis* biofilm was determined by calculating the amount of bacteria that survived post-exposure of the test material.

GSE solution used was Uzum Cekirdegi Ekstrakti, Immunat, and the tannin level was checked using spectrophotometry. The results showed that the level of tannin in the GSE solution was 2.9%. The 2% CHX solution used in this study was sold under the name Consepsis (Ultradent). *E. faecalis* American Type Culture Collection (ATCC) 29212 was obtained from KWIK-STIK™.

E. faecalis ATCC 29212 was evenly applied on brain heart infusion agar (BHIA), incubated at 37°C for 24 h, and subsequently inoculated into 10 mL of sterile saline using an ose needle. The density of the suspension was standardized using McFarland standard 0.5 to obtain 108 CFU/mL. The cellulose nitrate filter membrane (porosity, 0.2 µm; diameter, 13 mm; Whatman, Whatman International Ltd., Maidstone, UK) on BHIA was subsequently covered with 25 µL of bacterial suspension and incubated at 37°C for 72 h under aerobic condition (Fig. 1).

After incubation for 72 h, the membrane was aseptically transferred from BHIA into an Eppendorf tube containing 1 mL phosphate-buffered saline (PBS) to release bacteria that were not firmly attached to the membrane (planktonic bacteria). Next, each membrane was transferred into three Eppendorf tubes with 1 mL of PBS (control) solution, three Eppendorf tubes with 1 mL of GSE solution with 2.9% tannin content, and three Eppendorf tubes with 1 mL of 2% CHX solution and incubated at 37°C for 10 min under aerobic condition.

All membranes of the test and control groups were rinsed 3 times with 1 mL of PBS to neutralize and stop the activity of the antibacterial agents. Next, the last Eppendorf tube with the membrane was placed on the vortex machine for 2 min to obtain a bacterial suspension. The membrane was then aseptically removed from within the tube (Fig. 2).

Propidium monoazide (PMA) was added to the Eppendorf tube containing 100 µL of the bacterial suspension to obtain a final concentration of 100 µM and incubated at 4°C for 10 min in a dark room. Subsequently, the Eppendorf tube was horizontally placed on dry ice and exposed to 600 watts of halogen rays for 20 min at a distance of 20 cm.

Sample homogenization was performed for 10 s using a vortex machine, and centrifugation was performed at 10,000 rpm for 3 min or until it dissolved. Following centrifugation, the supernatant was discarded using a micro-pipette to leave only the natant inside the microcentrifuge tube. The entire natant was added to 200 µL of InstaGene™ Matrix and homogenized over hot plate using a magnetic stirrer.

Next, the sample was incubated in a water bath at 56°C for 30 min and homogenized using a vortex machine for 10 s. The tube was inserted into the thermoblock at 100°C for 8 min. This procedure was repeated 3 times.

Further, the sample was homogenized using a vortex machine for 10 s and centrifuged at 12,000 rpm for 3 min until the sample solution separated into natant and supernatant. The supernatant was transferred into a new microcentrifuge tube and kept at 4°C for 24 h. When in microcentrifuge tubes, natant deposits were still visible, but the supernatant was transferred again into a new microcentrifuge tube and stored at -20°C.



Fig. 1: (a) *Enterococcus faecalis* American Type Culture Collection (ATCC) 29212™ bacterial preparations, (b) culturing of *E. faecalis* ATCC 29212™ on brain heart infusion agar, (c) colonies of bacteria formed after incubation at 37°C for 24 h, (d) collection of bacterial colonies using ose needles to be inserted in a reaction tube containing NaCl, (e) density adjustment according to McFarland standard 0.5

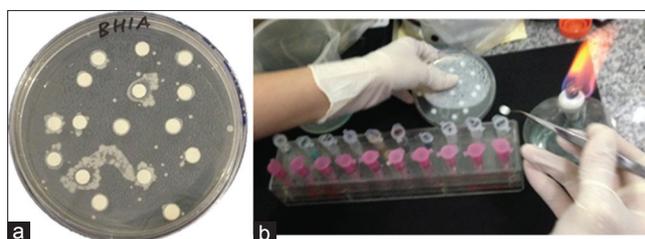


Fig. 2: (a) The biofilm formed after inoculation with *E. faecalis* at 37°C for 72 h, (b) bacterial biofilm is washed 3 times with phosphate-buffered saline (PBS) solution and is incubated in 2% chithexidine, 2% chlorhexidine, and PBS solution, which was used as a control, at 37°C for 10 min

Primers EF Gro ES-F and EF Gro ES-R were diluted using TE buffer. The ratio of EF Gro ES-F and EF Gro ES-R was 9:1. The diluted primers were homogenized using a vortex machine followed by a spin down machine. Next, the PCR mix was made as follow: (1) 1.5 mL microcentrifuge tubes were coated with aluminum foil; (2) volume of PCR mix was calculated by multiplying each mixture by the number of samples to be processed using RT-PCR. The required mixtures were 10 µL of Power SYBR® Green PCR Master Mix, 2 µL of universal primer 357F, 2 µL of universal primer 907R, and 2 µL of nuclease-free water, and (3) all PCR mix materials were combined in 1.5 mL microcentrifuge tubes that were coated with aluminum foil. Next, the mix was incorporated into the MicroAMP™ Fast reaction tubes with as much as 16 µL and added with 4 µL DNA samples. Finally, the mixture was homogenized using a micro-pipette.

MicroAmp™ Fast reaction tubes (8 tubes/strip) were covered with a MicroAmp™ Optical 8-Cap Strip for asepsis. PCR well plate was inserted into the step-one RT-PCR System (Applied Biosystems) and the parameters were adjusted as needed. RT-PCR quantitative cycle for total bacteria and preheat activation was performed at 95°C for 3 min, followed by denaturation at 95°C for 15 s (40 cycles), primary annealing at 55°C for 30 s, and elongation at 72°C for 30 s. Subsequently, the results were read on a computer screen (Fig. 3).

Data obtained were analyzed using SPSS 20.0 software. The number of viable *E. faecalis* from the control and test groups was analyzed for normality and homogeneity. One-way ANOVA was performed if the data



Fig. 3: (a and b) Addition of propidium monoazide and incubation in dark room, (c) exposure to 600 watts of halogen rays for 20 min, (d) detection and quantification of DNA of *Enterococcus faecalis* using real-time polymerase chain reaction

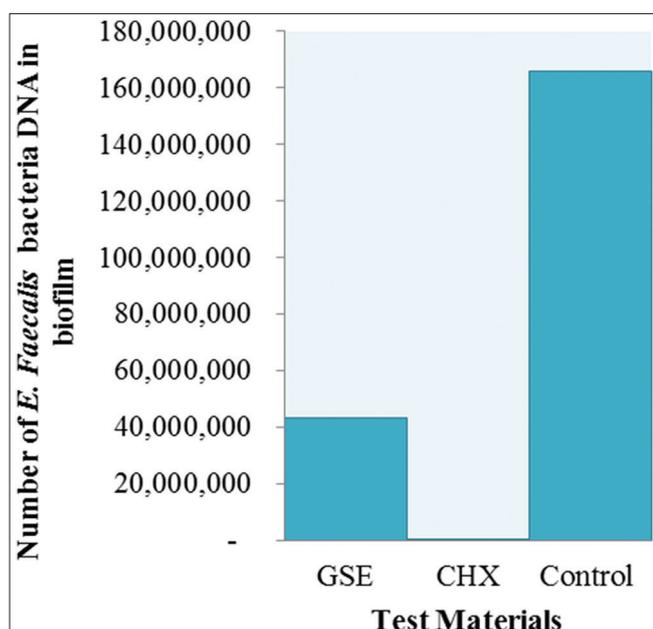


Fig. 4: Comparison of the mean DNA count of *Enterococcus faecalis* in biofilm (CFU/mL) in the GSE, CHX, and control groups. GSE: Grape seed extract, CHX: 2% Chlorhexidine

distribution was normal and homogeneous. Multiple comparison test with *post hoc* least significant difference was performed when there was a significant difference. Statistical analysis using Kruskal–Wallis non-parametric test and *post hoc* Mann–Whitney U-test was performed if the data distribution was not normal or homogeneous. $p \leq 0.05$ was considered statistically significant.

RESULTS

The results of this research quantified viable *E. faecalis* after exposure to GSE solution with 2.9% tannin and 2% CHX for 10 min. Non-parametric Kruskal–Wallis test was performed to assess the significance of bacterial yield because the data distribution was not homogeneous (Table 1).

The highest mean and standard deviation (SD) values were found in the control group, followed by the GSE and CHX groups. However, the SD values in the CHX group were greater than the mean values in the CHX group. Therefore, although the CHX group showed the lowest

Table 1: Mean of *E. faecalis* DNA count in biofilm after mixed with test material (CFU/mL)

Test material	n	Minimum	Maximum	Mean \pm SD
GSE	3	0.41×10^8	0.46×10^8	$0.43 \times 10^8 \pm 0.02 \times 10^8$
CHX	3	0.03×10^4	2.32×10^4	$0.86 \times 10^4 \pm 1.29 \times 10^4$
Control	3	1.33×10^8	2.11×10^8	$1.66 \times 10^8 \pm 0.40 \times 10^8$

GSE: Grape seed extract, CHX: 2% Chlorhexidine, *E. faecalis*: *Enterococcus faecalis*

Table 2: The significance of the antibacterial efficacy of each test group

Test group	CHX	Control
GSE	0.05*	0.05*
CHX		0.05*

*Significance level of $p \leq 0.05$ using *post hoc* Mann–Whitney U-test analysis.

GSE: Grape seed extract, CHX: 2% Chlorhexidine

mean of bacterial DNA, the data in the CHX group had the highest data deviation.

Fig. 4 shows the mean number of *E. faecalis* DNA in the biofilm in each test group. The highest mean DNA count was in the control group, followed by the GSE and CHX groups. Therefore, 2% CHX showed the highest antibacterial efficacy.

The difference in the amounts of DNA between the GSE and CHX groups, GSE and control groups, and CHX and control groups was significant ($p = 0.05$; Table 2).

DISCUSSION

The present study analyzed the antibacterial efficacy of GSE with 2.9% tannin against *E. faecalis* biofilm and compared the antibacterial efficacy of GSE solution with that of 2% CHX. The results of this study are consistent with that of the previous research by Angellina (2013) that analyzed the antibacterial efficacy of GSE as a root canal irrigation solution for smear-layer cleaning in a third apex of the root canal wall because the GSE solution was believed to increase the collagen cross-tooth strength [15]. The background of this study refers to the findings of Mageshwaran *et al.* (2012), who found that PAs in GSE provided antibacterial efficacy against *E. faecalis* in root canals [13].

At present, 2% CHX is the most effective root canal irrigation solution to remove *E. faecalis*; therefore, CHX was used as a standard in the present study. This is consistent with the Schafer and Bossmann findings, in 2005, which suggest that 2% CHX is effective against Gram-positive bacteria, such as *E. faecalis* [16].

E. faecalis ATCC 29212 with zero passage was used in the present study. The purpose of using zero passage is to avoid the risk of contamination in the subculture, errors during displacement and labeling, and occurrence of phenotypic changes or mutations that may occur during subculturing.

E. faecalis biofilm was used because there are several studies that prove that bacteria contained in a biofilm can withstand antibacterial agents compared to bacteria in planktonic form. The bacteria in the biofilm are attached to the root canal wall, isthmus, lateral root canal, and dentin tubules, thereby making it difficult to remove them with instrumentation alone [1].

The biofilm of *E. faecalis* was formed on cellulose nitrate membrane to obtain standardized growth for accurate antibacterial efficacy assessment [17].

RT-PCR was used to quantify the DNA. In conventional PCR, the detection and quantification of the amplified product are done at the last reaction

after the last PCR cycle, whereas, in RT-PCR, the amplicon is assessed at each cycle by analyzing the exponential phase of the reaction. PCR is one of the best methods in molecular biology (DNA-based) due to its high specificity, sensitivity, and speed [18]. Alvarez *et al.* (2013) suggested that the PCR method is widely developed because it is more sensitive to bacteria than conventional culture techniques. Conventional bacterial culture methods only detect bacterial cells that form colonies on nutrient media but cannot detect dead bacterial cells, viable but nonculturable (VBNC) bacterial cells, and bacteria that require special media for growth. According to Eswar *et al.* (2013), RT-PCR is a suitable method for detecting *E. faecalis* because *E. faecalis* has VBNC bacterial cells [19].

One disadvantage of PCR is that it cannot distinguish between living and dead cells; therefore, the DNA of dead cells is also readable on the PCR cycle. Therefore, this study used PMA in the sample. PMA is a propidium iodide (PI) derivative; PI colors dead cells by penetrating bacterial cell membranes that have lost their integrity. PI binds to bacterial DNA and emits fluorescence at certain wavelengths. This DNA modification renders it inactive during PCR, thereby differentiating between living cells and dead cells in the final quantitative results [20].

The presence of antibacterial efficacy in the GSE solution had been suggested by previous authors. According to Mageshwaran *et al.* (2012), GSE solution can inhibit the growth of *E. faecalis* [13]. In the group with a combination of CHX, calcium hydroxide, and GSE solutions showed a smaller zone of inhibition than the control group using agar diffusion methods. However, this research was different from the present study in terms of *E. faecalis* preparation and examination methods. Mageshwaran *et al.* (2012) used *E. faecalis* in planktonic form and the diffusion method, whereas the present study used *E. faecalis* in the form of biofilm and real-time PCR. To the best of our knowledge, no other study has been conducted using pure GSE solution, as done in this study.

The antibacterial mechanism of GSE is believed to be related to its chemical structure. PA interacts with proteins present in the bacterial cell membrane, resulting in the following three actions: The destruction of cell membranes, impairment of the proton motive force process, and inhibition of cell membrane enzymatic activity [21,22]. In addition, Xia *et al.* (2010) stated that the core structure of 3,4,5-trihydroxyphenyl that is found in the epigallocatechin monomer has a role in antibacterial action. The hydroxyl group and the double bonds present in this core structure bind proteins to the cell wall. Therefore, the antibacterial efficacy of the phenol compound depends on the number of hydroxyl groups and the degree of polymerization [23]. While the antibacterial mechanisms of CHX are related to positive molecules, these positive molecules can bind to negative ions, both in bacterial cell walls and in dentine hydroxyapatite. Bonding of bacterial cell walls causes nucleic acid leakage and bacterial cell lysis, whereas bonding in dentine causes CHX to be gradually released at a therapeutic level, which is known as the substantive nature [16]. GSE antibacterial properties are not proportional to CHX antibacterial properties due to the differences in their antibacterial mechanisms.

In this study, the antibacterial efficacy of GSE with 2.9% tannin could not exceed that of CHX because it is a natural material. Nevertheless, GSE has the advantage of good biological safety. GSE had been widely used as a health supplement due to its antioxidant properties [21]. In addition, Yamakoshi *et al.* (2002) reported that GSE is not mutagenic or toxic in studies conducted in mice [24]. This very low toxicity is important in endodontics because it can stimulate tissue regeneration and tissue healing.

The GSE solution used in this study contained 2.9% tannin, which is <5% concentration used by Mageshwaran *et al.* (2012). They used GSE preparations in the form of powder mixed with the water solvent. In addition, the GSE solution was combined with 2% CHX and calcium hydroxide. Differences in the preparations caused differences in the results obtained between theirs and the present study. In the current

study, the antibacterial efficacy of GSE solution compared with 2% CHX is not as good as that in previous studies.

CONCLUSION

The PAs (tannin) 2.9% in GSE provides antibacterial efficacy to *E. faecalis* biofilm characterized by decreasing the DNA count in *E. faecalis* biofilm after exposure to GSE. The antibacterial efficacy of GSE is lower than that of 2% CHX, possibly because the GSE solution is a natural ingredient and to match the antibacterial efficacy of 2% CHX, a high concentration of chemicals would be required. Although GSE has a lower antibacterial efficacy than 2% CHX, it is biologically safe and has potential as a root canal irrigation material.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare.

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