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.
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 was used from Uzum Çekirdeği 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 Consesis (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).

Propodium 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. The tube was inserted into three Eppendorf tubes with 1 mL of PBS to neutralize and stop the activity of the antibacterial agents. 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.

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
distribution was normal and homogeneous. Multiple comparison tests with post hoc least significant difference were 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≤0.05 was considered statistically significant.

RESULTS
The results of this research quantified viable Enterococcus faecalis after exposure to GSE solution 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 Magesharan et al. (2012), who found that BAs 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].

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 Magesharan et al. (2012), who found that BAs in GSE provided antibacterial efficacy against E. faecalis in root canals [13].

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

| Test material | n   | Minimum | Maximum   | Mean±SD            |
|---------------|-----|---------|-----------|--------------------|
| GSE           | 3   | 0.41×10³ | 0.46×10³  | 0.43×10²±0.02×10³  |
| CHX           | 3   | 0.03×10³ | 2.32×10³  | 0.86×10¹±1.29×10¹  |
| Control       | 3   | 1.33×10³ | 2.11×10³  | 1.66×10²±0.40×10³  |

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* |         |
| CHX        | 0.05* |         |

*Significance level of p≤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 Magesharan et al. (2012), who found that BAs 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-trihydroxyphenylalanine is found in the epigallocatechin monomer and has a role in antibacterial activity. 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.

REFERENCES

1. Siqueira J, Rocos I. Microbiology and treatment of endodontic infections. In: Hargreaves K, Cohen S, editors. Cohen’s: Pathways of the Pulp. St. Louis: Mosby Elsevier; 2011. p. 559-68.
2. Peters O, Peters C. Cleaning and shaping of root canal system. In: Hargreaves K, editor. Cohen’s: Pathways of the Pulp. St. Louis: Mosby; 2011. p. 41-56.
3. Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. Dent Clin North Am 2010;54:291-312.
4. Kakehashi S, Stanley HR, Fitzgerald RJ. The effect of surgical exposure of dental pulps in germ free and conventional laboratory rats. Oral Surg 1965;20:340-9.
5. Siqueira J, Rocos I. Primary Endodontic Infections. In: Siqueira JF, editor. Treatment of Endodontic Infections. Berlin: Quintessence Publishing; 2011. p. 115-7.
6. Stuart CH, Schwartz SA, Beeeson TJ, Owatz CB. Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. J Endod 2006;32:93-8.
7. Kandasawamy D, Venkatshababu N. Root canal irrigants. J Conserv Dent 2010;13:256-64.
8. Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza-Filho FJ. Comparative study of the antimicrobial efficacy of chlorhexidine gel, chlorhexidine solution and sodium hypochlorite as endodontic irrigants. Braz Dent J 2007;18:294-8.
9. Gernhardt CR, Eppendorf K, Kozlowski A, Brandt M. Toxicity of concentrated sodium hypochlorite used as an endodontic irrigant. Int Endod J 2004;37:277-80.
10. Onçaş O, Högör M, Hilimoğlu S, Zekioglu O, Eronat C, Burhanoglu D, et al. Comparison of antibacterial and toxic effects of various root canal irrigants. Int Endod J 2003;36:423-32.
11. de la Iglesia R, Milagro FJ, Campion J, Bosqüe N, Martínez JA. Healthy properties of proanthocyanidins. Biofactors 2010;36:159-68.
12. La VD, Howell AB, Grenier C. Denerby CR. Proanthocyanidins inhibit MMP production and activity. J Dent Res 2009:88:627-32.
13. Mageshwaran T, Ebenezar AR, Madhanamadhahula M, Kavitha S, Mahalaxmi S. Counteraction of reactive oxygen species and determination of antibacterial efficacy of proanthocyanidin and lycopene when mixed with calcium hydroxide and chlorhexidine mixture: An in vitro comparative study. J Conserv Dent 2012;15:337-41.
14. Ranjitha CY, Priyanka S, Deepika R, Rani GP. Antimicrobial activity of grape seed extract as Root Canal Irrigation Solution in Cleaning the Smear Layer. Thesis. Jakarta: Universitas Indonesia; 2013.
15. Ryan S. Chlorhexidine as a canal irrigant: A review. Compend Contin Educ Dent 2002;23:1-8.
16. Chai WL, Hannah H, Cheng SC, Sallam AA, Abdullah M. Susceptibility of Enterococcus faecalis biofilm to antibiotics and calcium hydroxide. J Oral Sci 2007;20:161-6.
17. Life Technologies. Real-Time PCR Handbook. Life Technologies; 2012.
18. EsWar K, Venkatshababu N, Rajaswari K, Kandasawamy D. Dentinal tubule disinfection with 2% chlorhexidine, garlic extract, and calcium hydroxide against Enterococcus faecalis using by real-time polymerase chain reaction: In vitro study. J Conserv Dent 2013;16:194-8.
19. Alvarez G, Gonzalez M, Isabal S, Blanc V, León R. Method to quantify live and dead cells in multi-species oral biofilm by real-time PCR with propidium monoazide. AMB Express 2013;3:1.
22. Perumalla A, Hettiarachchy N. Green tea and grape seed extracts potential applications in food safety and quality. Food Res Int 2011;44:827-39.
23. Sivarooban T, Hettiarachchy N, Johnson M. Physical and antimicrobial properties of grape seed extract, nisin and EDTA incorporated soy protein edible biofilm. Food Res Int 2008;41:781-5.
24. Xia EQ, Deng GF, Guo YJ, Li HB. Biological activities of polyphenols from grapes. Int J Mol Sci 2010;11:622-46.
24. Yamakoshi J, Saito M, Kataoka S, Kikuchi M. Safety evaluation of proanthocyanidin-rich extract from grape seeds. Food Chem Toxicol 2002;40:599-607.