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Influence of Extract Propolis on the Adherence of *Enterococcus faecalis* as a Candidate Root Canal Irrigation Solution

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ABSTRACT

Failure of endodontic treatment is commonly related to the presence of persistent microorganisms. *Enterococcus faecalis* can form a calcified biofilm in tough environmental conditions within the root canals. Biofilm-mediated infections can be caused by biofilm formation on tissues and biomaterial surfaces which are difficult to treat as a result of the increased antimicrobial resistance of biofilm bacteria. The bacteria that are close to a solid surface forms the initial and the most important step in the formation of biofilm. Propolis, natural bio product from bee contains tt-farnesol and apigenin that have mechanisms

for inhibiting adherence of bacterial biofilm. This study assessed the influence of extract Propolis on from the adherence of *Enterococcus faecalis*. This study was designed as post-test only control group laboratory experiment. The Propolis 8-16% was extracted by using maceration method and serial dilution. Susceptibilities to the test extracts were analyzed using the agar diffusion method and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC); the effect on bacterial adherence to a glass surface was also assessed. With the use of the one-way ANOVA with multiple comparisons, significantly fewer bacteria were found adhering to the extract Propolis. AlI treatment produced the maximum reduction in adherence of *E. faecalis* to smooth surface followed by 8-16%. Extract Propolis 14% show effectiveness in preventing adhesion of *E. faecalis*.

KEYWORDS

Clinical laboratory, Propolis, *E. faecalis*, adherence assay, experimental design, Indonesia

INTRODUCTION

Today, most of the problems faced in the field of dentistry are that almost all materials used in dentistry are of chemical import and has its side effects (Shahani & Subba Reddy, 2011; Kustarci, Altunbas, & Akpinar,2012).

Some researchers in Indonesia are looking for substitute chemicals by utilizing the ingredients of traditional crops which can be obtained from the natural environment in Indonesia. Indonesia has been known to have various types of local bees. One of the bees' by-products is the bee glue or propolis. Bee glue or propolis is known to have potential anti-microorganism which can be used for both pulp therapy in primary and permanent teeth (Ahuja & Ahuja,2011).

The main components of propolis are flavonoids and phenolic acids, including caffeic acid phenylesthylester (CAPE) which the compounds reaches 50% of the entire composition. Research shows that propolis extract and commercial propolis contained the same active compounds, which is flavonoids, phenolic, hydroquinone tannins, essential oils, steroids, saponins, and reducing sugars. In propolis extract also contained tt-farnesol (terpenoids) and apige (Riyanti, Hadidjah,&Iswari,2010; Agustrina, 2011; Koo *et al.*, 2003).

Some research suggest that the microorganisms that grow in biofilms can be two times to 1000-times more resistant to antimicrobial agents than planktonic

forms of the same organism (Mohammadi & Abbott, 2009). Research about the power of antibiofilm propolis extract against *E. faecalis* shows that antibiofilm activity in propolis Minimum Inhibitory Concentration (MIC) is 6% and Minimum Bactericidal Concentration (MBC) is 12% (Wahjuningrum, 2013).

E. faecalis is a persistent microorganism in root canals as gram-positive bacteria have the ability to invade into the dentin tubules and the canal walls. The area where *E. faecalis* can survive prolonged periods under unfavorable conditions for micro-organisms such as inadequate nutrition and high pH due to the provision of Calcium Hydroxide. *E. faecalis* produce collagen binding protein, such as angiotensin-converting enzyme (ACE) and Serine protease (SPR). The enzymes will improve adhesion to collagen dentin tubule wall. ACE improve ties *E. faecalis* against collagen type I.

Irrigation materials are used in root canal treatment for eliminating microorganisms of the root canal. Terms of irrigation material have power and capable of removing debris anti-microorganisms root canal. Irrigation material is expected to break the attachment between the microorganisms and the walls of the root canal that would eliminate microorganisms.

OBJECTIVE OF THE STUDY

The aim of this study was to analyze the effect of extract Propolis on the adherence of *Enterococcus faecalis.* In this case, the adherence of *Enterococcus faecalis* functions as a candidate root canal irrigation solution.

METHODOLOGY

Propolis samples and masseration samples of *A. mellifera* propolis were obtained from the Batu, Malang forest region of East Java state, Indonesia. Propolis contain resin (50%), wax (30%), essential oils (10%), pollen (5%), and organic components(5%) (Sakagami *et al.*, 2012). Resin contain flavonoid, fenol, andacid. One of phenol is Caffeic Acid Phenethyl Ester (CAPE) (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez,2008). It is important for antibacterial.

Dilution of Extract Propolis

Formulating solutions with various concentrations of propolis using dilution methods with sterile distilled water. The dilution formula is:

$$C1 \times V1 = C2 \times V2$$

C1 and V1 is an initial concentration and volume while C2 and V2 are the concentration and volume of dilutions (8,10,12,14% and 16%).

Preparation of *E. faecalis*

The media that are used are Brain Heart Infusion (BHI) obtained from microbiology laboratory of the Faculty of Dentistry, University of Airlangga.

E. faecalis bacteria derived from stock Laboratory of Microbiology, Faculty of Dentistry, University of Airlangga.

E. faecalis culture with 0.5 Mcfarland suspension and incubate for 18 hours at a temperature of 37C. Then standardized to 0.5 Mc Farland (1.5 x 108 CFU / ml).

Inhibition of Adherence of Growing Cells to a Glass Surface

The researchers grew the organisms at 37 °C 10% CO2 by an angle of 30° for 18 h in test tubes to assess the bacterial adherence to a glass surface, as detailed in Koo *et al.* (2003).The microorganisms (the same as described above) were grown in BHI broth plus 1% sucrose (w/v) containing sub-MIC concentrations of the test extracts or control (80% ethanol, v/v). The sub-MIC concentrations were relevant to the present study because they demonstrate bacterial growth. After incubation, the adhering cells were washed and resuspended in an ultrasonic bath (Vibracell, Sonics & Material Inc.). The amount of adherent cells was measured spectrophotometrically at 550 nm. Next, the researchers defined the concentration for total bacterial adherence inhibition (TBAI) and this was the lowest concentration, allowing no visible cell adherence on the glass surface (p, 0.05). Six replicates were made for each concentration of the test extracts.

The investigation protocol was approved by the Institutional Ethical Committee of Faculty of Dentistry, Airlangga University, Indonesia in compliance to research ethics.

This study was designed as a post-test only control group laboratory experiment. Extract Propolis 8-16% was extracted using maceration method and serial diluted. Using the agar diffusion method and by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), susceptibilities to the test extracts were analyzed. The effect on bacterial adherence to a glass surface was also assessed. Using the one-way ANOVA with multiple comparisons, significantly fewer bacteria were found adhering to the extract Propolis.

RESULTS AND DISCUSSION

E. faecalis bacterial adhesion barrier test by propolis extract is performed using Duarte *et al.*(2003) method with various concentrations of propolis extract and positive controls and a negative control, with the total number of samples is 35. *E. faecalis* bacteria were grown in BHI broth plus 1% of sucrose (w / v) containing propolis extract with a concentration 8,10,12,14% and 16%. Then the number of attached cells was measured using spectrophotometry at a wavelength of 550 nm.

In the preliminary research, the concentration of propolis extract was 8%. It turned out that the number of attached bacteria was reduced by 90% that is the positively controlled number of bacteria. Research is performed back by using the concentration of propolis extracts to range between 8% and 16%. namely the concentration of 8%, 10%, 12%, 14% and 16%.

Before the analysis test of the differences between the concentration of propolis extract against the number of bacteria attached, normality test was performed in each group.

In Group I (33%), in group III (83%) and group V (17%) of the specimens exhibited very few and scattered.

Table 1. Statistical analysis

Extract Propolis	N	(Mean) (x 108)	Standart Deviation (SD)
16%	5	0.0	0.0
14%	5	0.025	0.015
12%	5	0.097	0.013
10%	5	0.135	0.035
8%	5	0.115	0.018
control media	5	0.00	0.00
controlPositif	5	1.500	0.069

Based on the table and the picture above shows the results of the average number of bacteria *E. faecalis* has the least propolis extract contained in the group of 16% with a mean of 0, which means there is no adhesion *E. faecalis* bacteria on the surface of the glass wall. The concentration of propolis extracts can reduce the amount of bacteria to be very minimal *E. faecalis* is in the group of 14% propolis extract.

Table 2. Normality test result

Concentration	Asymp.Sig (2-tailed)	normal
14 µg/ml	0,860	Normal
12 µg/ml	0,702	Normal
10 µg/ml	0,841	Normal
8 µg/ml	0,931	Normal
control (+)	0,542	Normal

All datashowen normally with p>0,05

Table 3. The result of differences between groups

Concentration	**14 µg/ml**	**12 µg/ml**	**10 µg/ml**	**8 µg/ml**	**control (+)**
14 µg/ml		0.041*	0.001*	0.007*	0.000*
12 µg/ml	0.041*				0.000*
10 µg/ml	0.001*				0.000*
8 µg/ml	0.007*				0.000*
control (+)	0.000*	0.0000*	0.000*	0.000*	

*the mean diffrence is significant at the 0,05 level

The results were analyzed using statistical analysis SPSS version 17 for Windows. Propolis extract concentration of 12 ug / ml, 10 pg / ml, and 8 ug / ml showed no significant differences.

From the previous data, the researchers determined the concentration of extract of propolis 16 mg/ml and assured that there were no bacterial adhesions. However, the concentration, as presented in Table 3, shows significant differences between the group extracts of propolis 14 mg/ml, that is other groups and positively controlled (p-value <0.05). There are also clear differences between the positive control group and a group of other propolis extracts. However, among the group of propolis extract bacterial adhesions but there is still very little so that the concentration of 14 ug / ml is the minimum concentration of propolis extracts can inhibit bacterial adhesions *E. faecalis*. Measurement results were analyzed by using One-way ANOVA, followed by post-hoc Multiple Comparison Test. One-way ANOVA test is used to detect differences in the data group later and then with Post-Hoc Multiple Comparison Test to determine which data group has a significant difference.

Propolis extracts have antibacterial activity against attachment *E. faecalis* with collagen on dentin tubule wall through the active ingredient of apigenin and tt-farnesol.

Biofilms consist of various species of bacterial cells attached to the surface and bind to form a series of matrix. Biofilm formation begins from adhesion or bonding of bacteria on the surface. Some species of bacteria can form biofilms in various conditions. Certain environmental signals that cause different bacteria form biofilms. Environmental conditions include nutrients, pH, osmolarity, concentration of O2 and proximity to the surface. The population density may also trigger the formation of biofilms.

Attachment or adhesion can occur through a variety of mechanisms and involve both reversible and irreversible stages. The first one is reversible attachment and is usually preceded by hydrophobic interactions, electrostatic interactions or van der Waals bonding and can be affected by temperature or bonding hydrodynamic (Hubble, 4Hatton, Nallapareddy, Murray, & Gillespie, 2003). This activity is usually enough to make the bond bacteria and is usually followed by a phase of irreversible attachment that begins with a certain host cell or bacterial cell receptors. Adhesion proteins and receptors, in general, is clinically associated with biofilm-forming bacteria and is associated with early attachment, also facilitates congregative interaction of bacteria at the same or different species. Cell surface proteins such as pili, flagella or fimbrae was an adhesion protein and can bind to specific receptors or form a bond with the surface hydrophobic (O'Toole, Kaplan, & Kolter,2000). The attachment of bacteria to surfaces and biofilm formation are also influenced by the physical properties of the surface. Rougher surface generally occurs greater bacterial colonization as well (Donlan, 2000).

E. faecalis are attached to the wall surface or root canal dentin tubules. *E. faecalis* produce collagen binding protein, such as angiotensin-converting enzyme (ACE) and Serine protease (SPR). The enzymes will improve adhesion to collagen dentin tubule wall. ACE increase the binding of *E. faecalis* against collagen type I and IV in the dentin tubules and the canal wall. *E. faecalis* can survive in much time without any nutrient availability as it can derive nutrients from hyaluronan, which is converted by enzymes hyaluronidase. Moreover, it derives energy sources from the dentinal fluid even in a well-sealed root canal system. Under stress, *E. faecalis* produces aggregation substance (AS) which promote bacterial adhesion. Lipoteichoic *E. faecalis* acids protect against lethal condition, cytolysin, US-48 and bacteriocin which inhibit bacterial growth. Cytolysin destroys cells such as

erythrocytes, PMN cells, macrophages and kills gram-positive microorganism. This explains the shift from gram-positive to gram-negative one. Persistent *E. faecalis* can trigger an inflammatory reaction and may start periradicular injury.

Propolis that are proven to have antimicrobial effect is a natural substance that can inhibit bacterial adhesions *E. faecalis* at certain concentrations. Such an effect is due to the content of apigenin and tt-farnesol contained in propolis. The researchers looked at the effects invitro active ingredients of propolis extracts have a role to adherent activity, i.e., apigenin and tt-farnesol (Koo*et al.,* 2003). Apigenin and tt-farnesol arenon-toxic materials both in vitro and in vivo. To reduce the number of production glucan, many experts recommend the use of natural materials than broad-spectrum antibacterial material because it will affect the normal flora of the oral cavity.

Apigenin has a role for inhibiting the activity GTF GTF B and C, but apigenin does not have antibacterial properties against Lactobacillus acidophilus. Apigenin inhibits the activity of GTF GTF B and C. By influencing the activity of GTF GTF B and C, apigenin effectively inhibits the synthesis of insoluble glucan. Apigenin is a therapeutic substance that affects the enzyme activity of GTF without antibacterial activity. Apigenin is a potent non-competitive inhibitor of the activity of GTF B and C. Inhibition of the enzyme glucosyltransferase resulted in Lactobacillus acidophilus can not be attached to the tooth surface so that no changes in glucose, especially sucrose into glucan (Koo et al., 2003).

The tt-farnesol shows the barriers during the growth and metabolism of *E. faecalis* by damaging the cell membrane of bacteria, thus affecting glucan synthesis process. tt-farnesol affect the permeability of the cell membrane. Glucan synthesis by inhibiting the activity of tt-farnesol on propolis extracts are more a result of the effects on the cell membrane as compared to the activity of the enzyme, which is an inhibitor tt-farnesol GTF bad. The chemical structure and lipophilic properties tt-farnesol which supports localization of the membrane, causing changes in cell membrane permeability and fluidity. This membrane will cause damage to the cell membrane, not only reduces the bacterial metabolism, but also affect the synthesis of glucan by *E. faecalis*. By decreasing the adhesion, it can reduce *E. faecalis* glucan synthesis on the surface of the tooth root canal. *E. faecalis* bacterial accumulation on the surface of the tooth root canal would be decreased so that the process of biofilm formation also decreasing.

The results of this research and data analysis showed that the minimum concentration of propolis extracts to inhibit the attachment of bacteria glucan (Minimum Inhibition Concentration, MIC) *E. faecalis* is 14 ug/ml, in which

there is tiny adhesion of bacteria on the surface of the glass as compared to other concentrations. While the effective concentration of propolis extracts can eliminate all bacteria of *E. faecalis* (Minimum Bactericidal Concentration, MBC) which is at a concentration of 16 ug / ml, seen from the absence of attachment of bacteria on the surface of the glass.

CONCLUSIONS

Extract Propolis 14% is effective in preventing adhesion of *E. faecalis.* For that reason, it is important to have an effort of preventing *E. faecalis* that is, in fact, a persistent microorganism in root canals as gram-positive bacteria that invade into the dentin tubules and the canal walls. It is also important to improve adhesion to collagen dentin tubule wall. ACE improve ties *E. faecalis* against collagen type I. Most importantly, it is the time to benefit the natural resources of having the *E. faecalis* that can produce collagen binding protein, such as angiotensin-converting enzyme (ACE) and Serine protease (SPR).

RECOMMENDATIONS

The study hopes that further research will open up opportunities to take advantage of Extract Propolis 16% as a potential irrigation solution.

TRANSLATIONAL RESEARCH

The outcome of this study entitled *"Influence of Extract Propolis on the Adherence of Enterococcus faecalis as a Candidate Root Canal Irrigation Solution"* would be translated into pamphlets and instruction manuals that will potentially be of assistance to dentists, hospitals and healthcare professionals in Indonesia.

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