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Antimicrobial Effectiveness of Propolis and Probiotics Combination as Root Canal Medicament against *Enterococcus faecalis*

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ABSTRACT

The application of intracanal medicaments is essential to support root canal sterilization against intrusive bacteria, which can easily penetrate and remain survive in the dentinal tubules. Root canal medicament is expected to penetrate tubules and eradicate the bacteria, leading to prognosis improvement of endodontic treatment. There is an upcoming consideration on using natural substance as an alternative root canal medicament, due to the side effects potency of chemical-based medicament. Propolis and probiotics are two natural promising antimicrobial and health beneficial substance to consider. This study aims to evaluate antimicrobial capacity of probiotics and with propolis combination as potential medicaments against *Enterococcus faecalis*. Eighteen extracted human second premolars were sectioned 8 mm below the CEJ. The dentin blocks were then prepared under the similar dimensional standards. *E. faecalis* was used in the dentin blocks preconditioning incubation for 12h. The samples were classified into six groups (n = 3). Group 1: untreated; Group 2: $Ca(OH)_2$; Group 3: $Ca(OH)_2$ + probiotics; Group 4: $Ca(OH)_2$ + 1% water-based propolis (WEP); Group 5: probiotics; and Group 6: 1% WEP + probiotics. The grinded dentin was collected and recultivated. The number of cultivated bacteria was further counted. The results showed that the CFU was significantly lower in all groups compared to the untreated, but not significantly different between groups. Although they were not significantly different, probiotics and combined treatments exhibited lower CFU than the sole $Ca(OH)_2$.
Keyword: water-based propolis; calcium hydroxide; premolar; probiotics; root canal medications

Introduction

Mechanical and chemical root canal cleaning procedures such as irrigation were commonly performed during root canal treatment. The procedure is purposed to remove the necrotic debris and eradicate the pathogenic intrusive bacteria. However, in some conditions such procedures could not be sufficient to achieve the goals completely, since the certain amount of bacteria remain in the unreachable complex of root canal structure.[1] This instance, if not treated, will lead to more severe tissue damage or, even, possible systemic disease development.[2]

The presence of some persistent root canal bacteria sometimes could remain to be found.[3–5] One of the common persisting bacteria might be found in the endodontic flare up condition is *Enterococcus faecalis*. Several

studies have suggested that this bacteria is profound as a critical species found during clinical assessment.[3,4,6] *E. faecalis* is adaptive type of root canal bacteria that have strong survival ability against root canal environments.[7] This bacteria has the ability to penetrate and survive in the dentinal tubules.[8]

Root canal medicament is intended to enhance the eliminating process of remaining bacteria in the root canal area. Several chemical substances that have been commonly used in dental practice, including chlorhexidine gluconate (CHX) and calcium hydroxide ($Ca(OH)_2$). However, resistance to endodontic bacteria to such medicaments are currently being reported by some studies. Several reports suggest that relative resistance tends to increase on oral bacteria over repeated CHX exposure.[9,10] On the other hand, oral bacteria is suggested to withstand alkaline properties of $Ca(OH)_2$.[11] Therefore, alternative and supportive root canal medicament could be an important option to consider.

It is widely known that several natural sources are reported to have antibacterial activity. Natural ingredients that can be considered for this purpose are propolis and probiotics. Significant inhibitory capacity towards pathogenic bacteria growth profile as the outcome of propolis treatment are also previously reported in considerable studies.[12–15] This is proposed to be related to its bioactive components, such as flavonoids and terpenes.[16] However, propolis extracts toxicity on human tissue still become a concern. The widely used extracts are from hydroalcoholic extraction methods, and are reported to have cytotoxicity effect on cell. They decrease the viability of certain normal cell lines upon treatment.[17,18] concerning this matter, the extraction method should be changed to achieve the desirable outcome. One to consider is water-based extraction, since Rocha et al.[14] finds that water extract of propolis shows not only pronounced antibacterial effect, but also considered safer for normal cells.

Interestingly, probiotics have also been proposed to fit in with the desired characteristic as medicament alternatives, with minimal harmful issue for human usage.[19] They are reported to have inhibitory activity against pathogenic bacteria. Several commonly and commercially used probiotics are coming from genera *Lactobacillus, Bifidobacterium,* and *Saccharomyces*.[20,21] Findings from previous studies suggested that these probiotics may act on altering the growth of pathogenic bacteria through production of antibacterial substances.[22,23] Supernatant of *Lactobacillus* species show significant bacterial inhibition against several species of oral pathogenic bacteria.[23,24] The utilization of probiotics supernatant is thought to be safer for medicament than the whole culture compartments. Bermudez-Brito et al.[25] reported that the direct exposure of probiotic living bacteria toward dendritic cells results in significantly higher pro-inflammatory cytokine production compare to the supernatant treatment.

Propolis derivative product can be used solely or in combination with other substances to improve the antimicrobial capacity. The previous study showed that propolis extract combination with commonly used intracanal medicament such as $Ca(OH)_2$, may increase the antibacterial property of the root-canal medicament[26–28] without having pronounced toxicity effects.[29] This combination improve the antibacterial capacity of $Ca(OH)_2$, since it was reported to has low infusibility and solubility into the dental tubules, where these condition may also lead to less antibacterial capacity.[30] The combined of both propolis with probiotics also promote to the stronger inhibitory capacity against root canal pathogenic bacteria,[31,32] without significant propolis effects on the probiotic bacteria growing profile until a certain concentrations.[33,34]

According to some previous findings, it could be essential to evaluate the effect of local propolis extract and probiotics combination for potential alternative root canal medicament against *E. faecalis*. This study aims to determine the antimicrobial activity of 1% water-based propolis (WEP) and probiotics (*Lactobacillus acidophilus*) combination on the growth of *E. faecalis*.

Methods

This research protocol has met the feasibility and approval according to the Health Research Ethics Committee of The Faculty of Medicine and Health Sciences (088/EC-EXEM-KEPK FKIK UMY/X/2022). This research was conducted at the MMT Laboratory UMY. We used eighteen extracted human second premolars with good crown and root conditions. Dentin block preparation was performed by using a separating disc to cut an 8-mm-long strip below the cementoenamel junction (CEJ). Gates Glidden (GG) drill No. 1 with a slow speed handpiece was used to create the identical internal diameter of the root canals. The formed root canal was initially irrigated with 17% Ethylenediaminetetraacetic acid (EDTA) and 2.5% NaOCl. These steps were completed using syringe for smear layer removal and distilled water for chemical residues removal. The dentin blocks were then embedded in a cube mold filled with plaster gibs of paris. Dentin blocks were exposed with *E. faecalis* ATCC 29212, which previously were cultured at 37^{o}C for 24h and adjusted to the 0.5 McFarland standard, for 12h. The blocks were then irrigated with 5 mL saline post-incorporation treatment.

Dentin samples were collected using GG No. 3. The grinded dentin was transferred to 400 µL brain heart infusion (BHI) broth and incubated at 37^{o}C for 24h. Then, 50 µL of the obtained dentin suspension was placed into a BHI agar disc and further incubated for 24h. We then counted the number of the exhibited colony forming units (CFU/mL).

WEP was prepared from raw local propolis and was soaked in distilled water at room temperature for 48h. The suspension was filtered using filter paper and evaporated the solvent. The obtained material was then dissolved in the treatment media for 1% WEP.

L. acidophilus was cultured using BHI broth at 37^{o}C for 24h. We centrifuged the culture at 6000rpm to precipitate the bacteria. The supernatant was then collected and filtered by using 0.22 µm sterile syringe filter. The filtrate was stored in -80^{o}C prior to use.

The sample was divided into six groups (n = 3). Group 1: untreated (saline solution), Group 2: $Ca(OH)_2$, Group 3: $Ca(OH)_2$ + Probiotics (*L. acidophilus* supernatant), Group 4: $Ca(OH)_2$ + 1% WEP, Group 5: probiotics, Group 6: 1% WEP + Probiotics. The CFU/mL were then counted, and statistical analysis was carried out through the normality, homogeneity and followed by One-Way ANOVA (CI:95%) if the data were normally distributed.

Results and Discussion

Table 1. Shapiro-Wilk results

Treatments	Statistic	df	Sig.
Control	.891	3	.358
$Ca(OH)_2$	.809	3	.136
$Ca(OH)_2$ + Probiotics	.980	3	.729
$Ca(OH)_2$ + Propolis	.854	3	.251
Probiotic	.998	3	.909
Probiotics+ Propolis	.999	3	.935

Table 2. Data homogeneity results

Levene Statistic	df1	df2	Sig.
1.846	5	12	.178

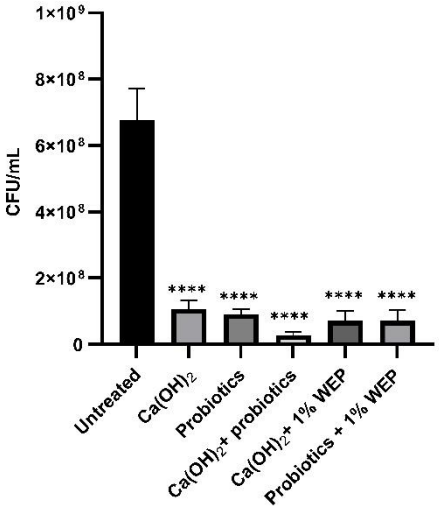
Table 3. One-Way ANOVA results

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	917667.593	5	183533.519	30.928	.0001
Within Groups	71209.947	12	5934.162		
Total	988877.540	17			

Table 4. Post Hoc results (LSD)

I vs J	Mean Difference (I-J)	Sig.
C vs $Ca(OH)_2$	569.33333*	.0001
C vs $Ca(OH)_2$ + Prob.	648.46667*	.0001
C vs $Ca(OH)_2$ + Prop.	604.60000*	.0001
C vs Prob.	585.06667*	.0001
C vs Prob. + Prop.	604.33333*	.0001
$Ca(OH)_2$ vs $Ca(OH)_2$ + Prob.	79.13333	.232
$Ca(OH)_2$ vs $Ca(OH)_2$ + Prop.	35.26667	.585
$Ca(OH)_2$ vs Prob.	15.73333	.807
$Ca(OH)_2$ vs Prob. + Prop.	35.00000	.588
$Ca(OH)_2$ + Prob. vs $Ca(OH)_2$ + Prop.	-43.86667	.499
$Ca(OH)_2$ + Prob. vs Prob.	-63.40000	.333
$Ca(OH)_2$ + Prob. vs Prob. + Prop.	-44.13333	.496
$Ca(OH)_2$ + Prop. vs Prob.	-19.53333	.761
$Ca(OH)_2$ + Prop. vs Prob. + Prop.	-.26667	.997
Prob. vs Prob. + Prop.	19.26667	.765

*C: untreated, Prob.: probiotics, Prop.: Propolis



The obtained data from all groups indicated that it was normally distributed (Table 1) test ($p>0.05$) and homogenous (Table 2) test ($p>0.05$). Thus, the data were further analyzed by using One-Way ANOVA. Table 3 showed that the treatments groups showed $p<0.05$ ($p<0.0001$), with $F_{tabel(5,12)}=3,11$ ($F_{cal}>F_{table}$; reject the null), it suggested that there was a significant difference among the treatment groups according to the obtained *E. faecalis* growing profile.

Based on the post hoc analysis, it also showed that there was a significant difference between the control group and all treatment groups (Table 4). The untreated group was significantly different from the rest groups ($p<0.05$). In the contrary, the treatments group were not significantly different ($p > 0.05$).

According to Figure 1, the untreated group has the highest CFU (676.67 x 10^6 (CFU/mL) of *E. faecalis* compared to all groups. While the CFU of all treatment's groups were not different among each other. However, probiotics and all the combination treatments were resulting in lower CFU level compared to sole $Ca(OH)_2$. However, sole probiotics had the lowest CFU (28.20 x 10^6 (CFU/mL) among the treatments.

According to the results, we found that the antimicrobial activity of probiotics and combined treatments on the growth of *E. faecalis* bacteria were statistically significant, compared to the untreated. All the treatments (probiotics and combined substances) significantly lowered the CFU of *E. faecalis* growing capacity. These findings were

consistent with previous studies. As expected previously, $Ca(OH)_2$ showed prominent antibacterial activity against *E. faecalis* in *in vitro* study. The similar result was also reported by Sangalli et al.[35]

Furthermore, our results also showed that probiotics supernatant could inhibit *E. faecalis* solely without any combination with other substances. This was also been reported before by Shaaban et al.[24] Probiotics are capable of producing antibacterial protein such as bacteriocin, which may inhibit the proliferation of intrusive bacteria and some other bacteria species.[36]

The combination of $Ca(OH)_2$ with probiotic indicated slightly better inhibitory capacity against *E. faecalis* than $Ca(OH)_2$. $Ca(OH)_2$ has known to have the ability to produce hydroxyl ions leading to cytoplasmic rupture of the bacteria, and interestingly this mechanism of action seemed to be supported by the presence of probiotics derivative products.[37] Corroborate with the previous result, the combined $Ca(OH)_2$ with propolis also showed slight improvement on inhibiting the growth of *E. faecalis* compare to the sole $Ca(OH)_2$. This improvement of $Ca(OH)_2$ antibacterial capacity was also reported by Elsayed et al. with different source of propolis and preparation procedures.[37] The phenolic and terpenoid compound of propolis extract may disrupt the bacterial capsule, cause cellular leakage, and affect the bacterial proteolytic and metabolic properties.[13,15,38] Finally, the combination of probiotics and propolis showed effective antibacterial activity against mono-cultured *E. faecalis*. Similar findings with different type of propolis, probiotic bacteria and treatment procedures were reported in a study by Ucar.[32] The composition of each components may act synergistically in inhibiting the proliferation of *E. faecalis*.

Conclusion

The sole $Ca(OH)_2$, probiotics, and all the combined treatments have pronounced inhibitory capacity against *E. faecalis*. In our findings, combination of $Ca(OH)_2$ with either two natural antibacterial derived-substances have slightly enhanced antibacterial capacity.

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