Metabolism-independent phenomenon in ethanolic propolis inhibitory capacity towards *enterococcus* spp proteolytic activity

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ABSTRACT

Background: *Root canal bacteria produce many virulence factors which are responsible for endodontic pathological states. Bacteria are assumed to utilize energy from bacterial cell metabolism activity for producing these virulence factors. Propolis extracts are commonly reported to have antibacterial abilities against dental pathogens. The purpose of this study is to investigate the possible correlation between bacterial proteolytic and metabolism activities under the treatment of ethanolic extract of propolis (EEP).*

Method: *The 0.00125%; 0.05%; 0.1%; 0.2%; 0.4%; and 0.8% ethanolic propolis were prepared for recovery rate confirmative procedure, proteolytic, and metabolism activity assay, with 2% of chlorhexidine gluconate (CHX) was used as a positive control. The bacteria were cultured in brain heart infusion (BHI) media after EEP treatment. Bacterial suspension was initially prepared in broth culture dilution with BHI media, followed by the gelatin liquefaction measurement for proteolytic assay. Phenol-red and arginine dehydrogenase enriched media for observing both carbohydrate and arginine metabolism activities, respectively, in the clinical Enterococcus spp. and E. faecalis ATCC 29212.*

Result: *The recovery rate of the bacteria was not terminated after several EEP treatments. Proteolytic activity of the bacteria was likely decreased in several EEP treatments. EEP tended to affect the carbohydrate and arginine metabolism of the bacteria in certain fashions.*

Conclusion: *This study suggested that the EEP treatment affected both proteolytic and metabolism activity in negative regulation tendencies.*

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INTRODUCTION

Endodontic treatment is a challenging procedure due to the possibility for reinfection and developing periapical pathologic condition after the treatment process. This pathologic state could be affected by several factors such as chemical, iatrogenic error, filling problem, incomplete sterilizing procedure and persistent microorganisms (microbiome dysbiosis concern).[1,2] One of bacteria which is presumably found to be resistant against root canal medicaments is *Enterococcus faecalis.*[3,4] *E. faecalis* is a bacterium that is involved in persistent endodontic infections with a prevalence of 24% to 77%.[5] *E. faecalis* is found in dentinal tubules radicular area in both colony or solely form.[6]

E. faecalis is a gram-positive biofilm producing bacterium,[7] and commonly to be found in colony with consist of cocci or chain forms of the bacteria.[8] These bacteria in a biofilm matrix are able to confront stresses derived from antibiotic application and highly alkaline environment.[7] On the other hand, *E. faecalis* produce virulence factors in order to confer damage on endodontic tissue.[8] One of the factor is gelatinase, a metalloprotease which may cleavages peptides component in endodontic surroundings,[9] and is associated with microbial adhesion to the dentin surface.[10] The gelatinase expression by *E. faecalis* is known to be important in the collagen hydrolysis mechanism which plays an critical role in initiating periapical pathogenesis, attract nonspecific immune cellular system, and subsequently lead to tissue destructions.[11] Some studies have mentioned that *E. faecalis* has been resistant to some antibiotics such as vancomycin, metronidazole, clindamycin, and tetracycline.[8]

E. faecalis uses carbohydrates and host serum components from dentin and its tubules as precursors for its metabolism activity.[8] Originally, *E. faecalis* is able to produce energy from those substances in both anaerobic and aerobic atmospheric conditions. Catabolism of carbohydrates in bacterial cells is performed through various metabolic pathways, such as glycolysis, oxidative pentose-phosphate, and *Entner-Doudoroff pathway*. In anaerobic conditions, energy is obtained from carbohydrate metabolism and lactic acid fermentation, whereas in aerobic condition, oxidative phosphorylation may occur with the availability of hemes and oxygen in the surrounding environment. In addition to carbohydrate based metabolism, *E. faecalis* may also come with arginine as a source of lower energy acquisition, compared to glycolysis.[12] Catabolism of arginine could be performed via arginase pathway and arginine deiminase or also known as ADI pathway.[13]

In the arginase pathway, the enzyme hydrolyses arginine into glutamate, then will further be converted into α-ketoglutarate so it can enter in the citric acid cycle to produce ATP. In the ADI pathway, arginine is hydrolyzed to citrulline. Then, it will be altered by ornithine carbamoyltransferase (ARCB) into carbamoyl phosphate and ornithine.[14] Eventually carbamoyl phosphate is used for ADP phosphorylation in a catalyzed reaction by the carbamate kinase enzyme that generates ATP as energy and residual products in the form of CO_2 and NH_3.[15]

Propolis has many active biological compounds,[16] and its extract shows inhibitory effects against bacteria.[17,18] Our previous report suggests that ethanolic extract of propolis (EEP) from *Apis trigona* could inhibit *E. faecalis* growth significantly.[19] This antibacterial effect of propolis extract is thought to be related to the active biological compounds such as terpenes and phenolic substances within the extract. Those substances are assumed to impair the membrane structure of bacteria, resulting in altered cytoplasmic components and leading to cell death.[20,21] According to the previous evidences, propolis and its extracts have the potential in inhibiting pathogenic oral bacteria from disease development.

To generate efficient and effective oral medicament, it is a necessity to understand the mechanism of inhibitory effects of propolis against oral microbial pathogens. Therefore, according to the above explanation, this study aims to examine the effect of EEP on the activity of carbohydrates and arginine metabolism of *E. faecalis* during its proteolytic activity.

METHODS

This research protocol was approved by the Ethical Committees Faculty of Medicine and Health Sciences Universitas Muhammadiyah Yogyakarta (386/EP-FKIK-UMY/VIII/2018).

Raw propolis material from *Apis trigona* was obtained from a local apiary in Nglipar, Gunung Kidul district D.I. Yogyakarta and extracted using maceration technique. We used several concentrations of EEP: 0.00125%; 0.05%; 0.1%; 0.2%; 0.4%; and 0.8% for both observing the most effective inhibitory capacity and also obtaining the proteolytic inhibitory activity profile. We used three different concentrations of 0.00125% and 0.4%, since they showed significant inhibitory effects on *E. faecalis* and *Porphyromonas gingivalis*, respectively[19] as previously reported, and 10% as the extended higher concentration for the pre-assumed maximum effect in inhibiting recovery rate, carbohydrate and arginine metabolism of the bacteria. 2% of chlorhexidine (CHX) was used as antibacterial positive control against the bacteria.[22,23] *E. faecalis* ATCC 29212 in this study were originally provided by the Health Laboratory (BLK) of Yogyakarta while the clinical sample (previously had been identified as *Enterococci*) were obtained from patient’s root canal under endodontic treatment at the academic dental hospital (RSGM), Universitas Muhammadiyah Yogyakarta.

Each sample was cultured in 25 ml of brain heart infusion (BHI) broth (Thermo Scientific™ Oxoid™) and incubated for 24 hours (h) at 37°C. For confirmation purpose, the clinical bacteria were plated in Slanetz-Bartley (Thermo Scientific™ Oxoid™) agar media for 24h at 44°C. *Enterococcus* bacteria were characterized by dark red dots on the selective media. Single colonies were selected and inoculated in BHI broth for 24h at 37°C. Suspect clinical bacteria inoculated into aerobic and anaerobic arginine dehydrolase broth media. The final confirmation was using mannitol metabolic test[5] for clinical bacteria with mannitol salt agar media (Thermo Scientific™ Oxoid™).

Recovery rate

200µl of bacterial culture were inoculated in EEP media with several different concentrations, as well as in positive and negative controls media. The cultures were incubated for 1h at 37°C. BHI broth was added to all the tubes after removal of media treatment and the bacteria cultures were incubated once more for 24h at 37°C. The optical density (OD) values of post treatment and after re-incubating were determined using Spectrophotometer UV Mini 1240 (Shimadzu) at 600nm.

Proteolytic analysis

Four groups of treatment: untreated, negative control using sterile distilled water, positive control using antibiotic ampicillin, and EEP were prepared. Each tube was filled with 4.8 ml BHI and 200µl of bacteria culture. The turbidity was determined according to the OD values obtained from spectroscopy analysis using Spectrophotometer UV Mini 1240 (Shimadzu).

For gelatinase assay, the cultures were added to the prefilled gelatine tubes. The tubes were then incubated in anaerobic jar at 35°C. The tubes were placed in the refrigerator for 1h prior measurement, and the gelatin liquefaction height was measured using the sliding caliper.

Metabolic Activity

To analyze the metabolic activities of carbohydrate and arginine, the cultures ~~of~~ bacteria were incubated in phenol red and arginine dehydrolase based-broth culture media for 24h and 48h, respectively, at 37°C. Color changes were observed qualitatively and then followed by quantification using ImageJ software 1.53o (NIH, Bethesda, MD, USA).

Statistical Analysis

Data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, USA). The normality test was conducted using Shapiro-Wilk, followed by ANOVA with Dunnet's as the post hoc analysis.

RESULTS

Recovery rate refers to a capacity of bacteria to resolve its population after the given treatment. Based on Figure 1, the data showed no significant reduction in resolving capacity after 1h treatment with either 0.00125%, 0.4% and 10% EEP on clinical bacteria recovery rate compared to the untreated group (p>0.9999). However, the significant reduction could be observed in the CHX 2% treated group (67.98 ± 2.02%, p=0.0042).

Based on Figure 1, several concentrations of EEP did not affect the bacteria recovery capacity, since they were not significantly different to the untreated group. The results confirm that the EEP-treated bacteria could re-grow after culture media replacement.

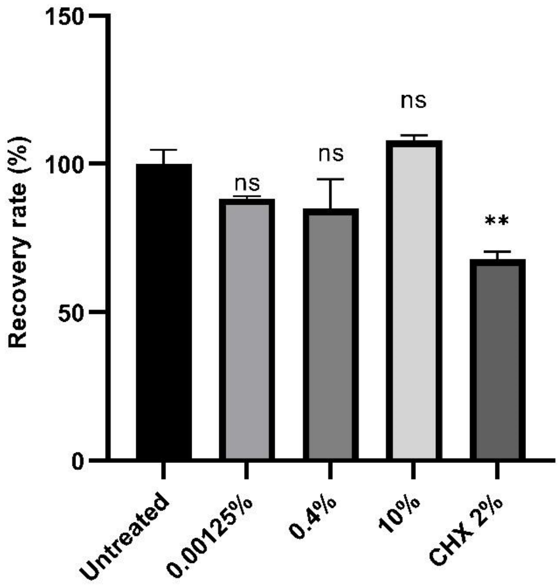


Figure 1. Bacterial recovery rate. *Enterococci* was treated using 0.00125%, 0.4%, 10% EEP solution, and positive control using 2% of CHX digluconate for 1h. The recovery rate was obtained from the OD difference between 24h post incubation BHI broth resuspension and the post 1h of each treatment (ΔOD_{600}). The ΔOD_{600} was divided by the negative control/untreated (BHI only) to obtain the percentage of recovery rate. Data were stated as mean ± standard error of the mean (SEM), with the significance level for each annotation: ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, *p<0.0001.

Based on Figure 2, it showed that the bacterial proteolytic activity decreased gradually along with increasing EEP concentration. Interestingly, the proteolytic activity increased in 0.8% EEP treatment. It seems that EEP has inhibitory capacity in bacterial proteolytic activity, which was gradually decreasing until a certain concentration. However, it can be inferred that the lowest bacterial proteolytic activity was in 0.4% of EEP compared to other concentrations.

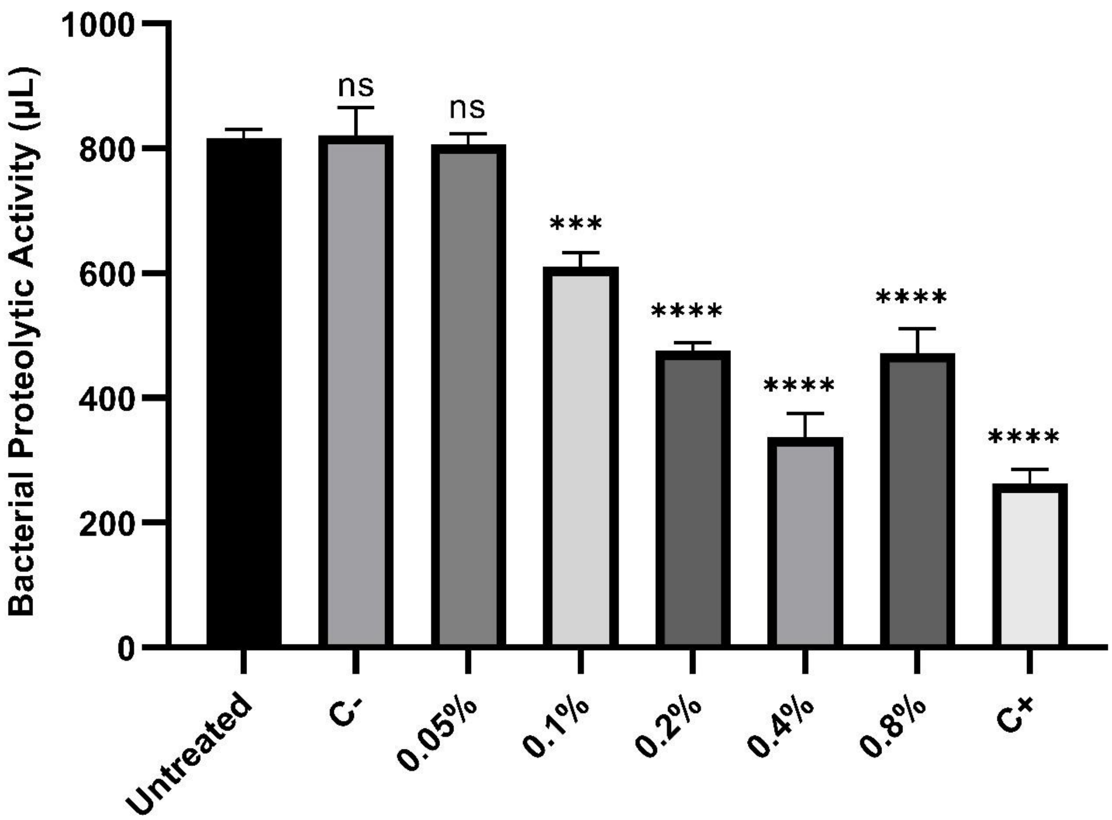


Figure 2. *E. faecalis* proteolytic activity. Means of liquefaction volume (µL); untreated (N) 815.8 µL; C+ (ampicillin) 263.2 µL; C- (distilled water) 821.3 µL; 0.8% EEP 471.8 µL; 0.4% EEP 336.7 µL; 0.2% EEP 475.5 µL; 0.1% EEP 610.8 µL; 0.05% EEP 806.5 µL. Proteolytic activity of *E. faecalis* described by liquefaction volume of gelatin agar in anaerobic jar. Data were stated as mean ± SEM, with the significance level for each annotation: ns=non-significant, *p<0.05, **p<0.01, ***p<0.001, *p<0.0001.

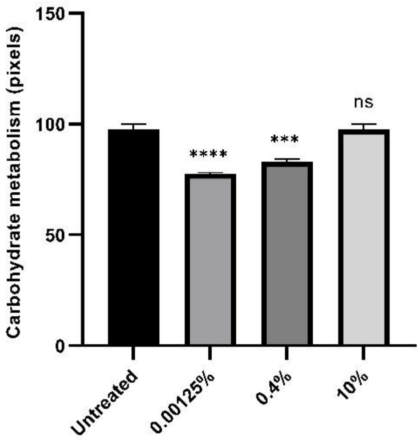
Table 1 showed that all the EEP-treated bacteria could maintain both carbohydrate and arginine metabolism despite the increasing EEP concentration, which was similar with the untreated group as a negative control. Only in 2% of CHX treatment that the bacteria showed no metabolism of the substances. Therefore, we proceed to the further analysis using EEP treatment.

Table 1. Metabolism activity – Qualitative

Treatment	Carbohydrate metabolism		Arginine metabolism	
	Enterococci	ATCC 29212	Enterococci	ATCC 29212
10% EEP	+	+	+	+
0.4% EEP	+	+	+	+
0.00125% EEP	+	+	+	+
CHX 2%	-	-	-	-
Untreated	+	+	+	+

According to Figure 3a-b, carbohydrate metabolism in both 10% EEP-treated ATCC 29212 and clinical bacteria showed the highest activity (97.387 ± 2.4120 and 99.743 ± 1.2808, respectively) and had similarity with the untreated group (p>0.9999). In Figure 3c-d there were also variances in bacterial arginine metabolism activity. EEPs were effective against sole species bacteria (Figure 3c). The arginine metabolism decreased gradually upon increasing EEP treatment, compared to the untreated group (p=0.0088, p=0.0034, and p=0.0006, respectively). Whereas, these results were different in multispecies bacterial colony in clinical bacteria. The arginine metabolism tended to increase in several EEP concentrations (Figure 3d).

Quantification data using ImageJ showed that in clinical sample with 0.00125%, 0.4%, 10% of EEP treatments compared to untreated in carbohydrate metabolism have significance values of p<0.0001, p<0.0001, and p>0.9999, respectively (Figure 3b), and on arginine metabolism have significance values of p=0.003, p=0.0228, and p=0.0775, respectively (Figure 3d). On the other hand, the ATCC 29212 with 0.00125%, 0.4%, 10% of EEP compared to negative control on carbohydrate metabolism have significance values of p<0.0001, p=0.0008, and p>0.9999, respectively, and on arginine metabolism have significance values of p=0.0088, p=0.0034, and p=0.0006, respectively.



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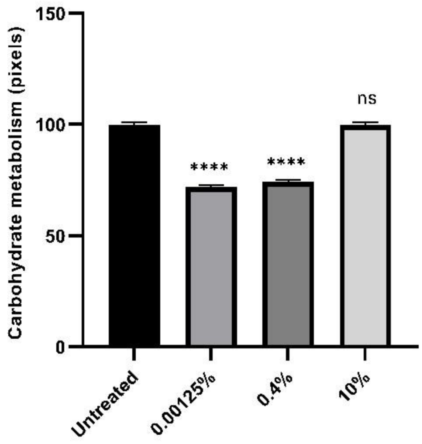

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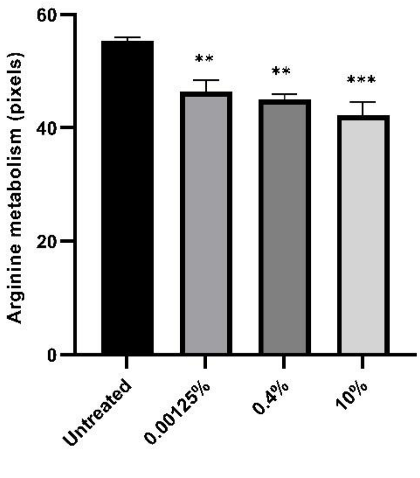

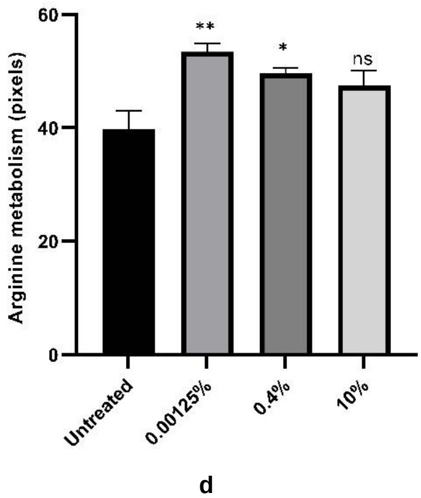

Figure 3. a, b) Carbohydrate metabolism profile of ATCC 29212 and *Enterococci*, respectively. c,d) Arginine metabolism profile of ATCC 29212 and *Enterococci*, respectively. Data were stated as mean ± SEM, with the significance level for each annotation, ns: non-significant, *p<0.05, **p<0.01, ***p<0.001, *p<0.0001

DISCUSSION

Recovery Rate

Based on the observation of the recovery rate depicted in Figure 1, it showed that the bacteria could overcome the EEP treatments to recover their growing profiles. Several concentrations of EEP had not yet affected the bacterial recovery capacity, though the tendency was shown. Even when the controlled-exposure of EEP treatment is eliminated and the bacteria get nutrients from basic culture media, the bacteria might be able to recover their original state of growing capacity.

This recovery test was a preliminary step, performed to obtain the equivalent number of bacteria within the sample. This equivalency would serve as a base condition for further experimental procedures.

Proteolytic Activity

Based on Figure 2, almost all concentrations of EEP could affect bacteria proteolytic activity. The most effective EEP concentration for inhibition of proteolytic activity could be observed at 0.4% EEP, compared with the other concentrations (0.8%; 0.2%; 0.1%; and 0.05%). EEP has flavonoid activities related to their ability in inhibiting proteolytic enzymes, one of which is through the molecular action of flavonoids to form complexes with proteins through non-specific forces such as hydrogen bonds and hydrophobic effects, as well as the formation of covalent bond.[24] Increasing number of hydroxyl groups can increase the bond between flavonoids to the enzyme, this provides an advantage in reducing enzymatic activity. The inhibition of flavonoids is related to the complexity of the flavonoid structure when interacting with enzymes.[25] In addition, the modification of glycosylation in flavonoids is also able to provide the similar effect, which can make flavonoids more interactive to bind to the substrate in the proteinase system, so that it may affect the enzyme-substrate interaction. With this glycosylation mechanism, it is suspected that EEP can bind to the substrate thereby inhibiting enzyme activity.[24]

Metabolism Activity

Based on bacterial metabolic activity on Table 1, it showed that *Enterococci* and ATCC 29212 maintained the metabolic activities of carbohydrate and arginine after the treatment with 0.00125%; 0.4%; and 10% of EEP, however, this was not shown in CHX-treated bacteria. According to Figure 3, it showed that there are variances on bacterial metabolic activity in several concentrations of EEP. 10% of EEP had similar level with the untreated group. It was probably due to the glucose residue that was reserved in EEP itself resulting in bacteria getting additional nutrients from EEP, which causes higher levels of carbohydrate metabolism and tends to overcome the antibacterial capacity and balancing the two opposite effects. On the other hand, there was a unique feature in arginine metabolism between both untreated clinical bacteria and ATCC 29212. The arginine metabolic activity of clinical bacteria seemed to increase in EEP treatments, but the ATCC 29212 showed the opposite. It was probably due to not only *E. faecalis* that doing their metabolism activity, but also some other species of root canal *Enterococci* in the sample may also be involved in arginine metabolism that have similar metabolism features to *E. faecalis*.

In our previous initial hypothesis, EEP was thought to affect the bacterial metabolism than it will block bacterial growth and proteolytic activity. Interestingly, in our findings, EEP seems not to affect the bacterial growth, but tends to inhibit proteolytic activity by reducing the synthesis of gelatinase for several concentrations. However, this did not seem to correlate with the effect of propolis on metabolic activities of the bacteria. Significant effect on proteolytic activity was only shown in 0.1% and higher EEP treatments, whereas EEP lower than 0.1% had significant effect on metabolic activities of bacteria. It seems that the inhibitory capacity of EEP is likely affecting the cell metabolic activity rather than inhibiting the population growth.

CONCLUSION

According to our findings, it could be concluded that EEPs may affect *E faecalis* proteolytic activity and their metabolism in an independent manner.

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