

A Challenge in Ethanolic Propolis Utilization from *Apis Trigona* as an Oral Antimicrobial Agent

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Abstract

Propolis is one of bee's product which harboring many biological compounds including flavonoid. Considering all biological aspects in oral medicament development, both benefit and biocompatibility issues must be confirmed. *Enterococcus faecalis* and *Porphyromonas gingivalis* are known to be the critical oral pathogenic bacteria which are responsible for endodontic failure and periodontal diseases respectively. The purpose of this study was to investigate the effect of ethanolic propolis compound from *Apis Trigona* towards bacterial growth and cytotoxicity. Propolis *Apis Trigona* was taken from local apiary in Nglihar subdistrict of Yogyakarta, Indonesia and was extracted using maceration procedure. *Enterococcus faecalis* and *Porphyromonas gingivalis* were used for bacterial growth evaluation, while fibroblast cells were used for cytotoxic analysis. One way Anova, Kruskal Wallis and Pearson tests were performed for statistical analysis. All treatments were performed in triplicate experimental design. The variances analysis showed $p=0.000$ and $p=0.009$ for the effect of ethanolic propolis which indicated potential results, while Pearson correlation coefficient showed the score -0.673 which indicated strong negative correlation. Taken together, these results suggested that the higher bacterial inhibitory capacity, the lower cells viability on the contrary. Further investigations are required for gaining the optimum benefit from propolis *Apis Trigona* in a higher biocompatibility.

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Introduction

Propolis or originally called bee glue is one of the bee's product which harboring many biological properties and play a critical role in protecting the bee hives against threatening microorganisms towards colonies.¹ There are a lot of reports showing the benefits from propolis biological components due to the existence of *polyphenolic*-derived substances such as *flavonoids*. *Flavonoids* group of substance has been observed in many studies in exhibiting a lot of biological activities such as an antioxidant, anti-inflammatory agent, anti-viral, anti-fungal,

and anti-microbial agent.² Some of *flavonoid*-derived organic compounds which have been reported in harboring anti-microbial capacity such as *caffeic acid phenethyl ester* (CAPE) and *galangin*³ and also other derivatives from flavonoids group such as *flavone*, *chalcone*, *flavanone*, *flavonol*, *flavan-3-ol* (*Cathecin*), and *flavolan* (*proanthocyanidin*) which belong to the 6 subclasses of *flavonoids* harboring antibacterial property.⁴

Oral cavity is commonly occupied by many species of oral microflora in a balance interaction. Disturbances in the balance of oral microflora interaction may lead to oral disease development such as caries and periodontal diseases.⁵ *Enterococcus faecalis* is one of the responsible bacteria in the failure of endodontic treatment which belongs to gram positive, facultative anaerobic bacterium and shows very persistent bacteria against highly extreme living environment.⁶ Among several properties of

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virulence factor owned by *Enterococcus faecalis*, capsular polysaccharide is one of potential factor that may contribute to the persistence of *Enterococcus faecalis* in the root canal.⁷ On the other hand, *Porphyromonas gingivalis* is a periodontopathogen bacterium that belongs to gram negative and obligate anaerobic bacteria which commonly related to the occurrence in periodontal diseases.⁸ Both failure of endodontic treatment and periodontal diseases still become the common problems and concerns among the dental issues.^{6,9} Understanding in bacterial inhibitory activities may support for controlling oral bacterial population and its growth ability through utilizing adequate antimicrobial agent.

Since propolis has been reported on harboring many biological properties that benefit for controlling oral bacterial population. Propolis compounds become one of interesting natural product for developing antimicrobial agent particularly against oral pathogenic bacteria. In order to be applied clinically, it has to be non-toxic to the host cells and tissues. Therefore, biocompatibility issue must be confirmed and solved before going to the clinical application.¹⁰ Since each bee's propolis product could be vary due to many factors including species and surrounding plantations. As far as the authors knowledge, the study of this local bee's propolis has not been reported yet. The purpose of this study is to confirm the antibacterial potency through bacterial growth inhibitory capacity and its cytotoxicity effect of the local propolis from *Apis Trigona*.

Materials and Methods

Raw propolis material from *Apis Trigona* (900gr) was obtained from local apiary in Nglipar, Gunung Kidul district of Yogyakarta, Indonesia and was extracted under maceration procedure using 8L 40 % ethanol as described previously¹¹ to get 100%ethanolic propolis compound material. *Enterococcus faecalis* (ATCC-29212) and *Porphyromonas gingivalis* (ATCC-33277) were provided by the district Health Laboratory Institution (BLK). Each bacterium was cultured in BHI broth for *Enterococcus faecalis* and tryptose phosphate broth for *Porphyromonas gingivalis* media using anaerobic jar 3.5L supplemented with anaerogen, at 37°C incubator. Human adult fibroblast cells (HDFa-Gibco C-013-5C, USA) were treated in 10% FBS supplemented-DMEM

culture media using 50mL culture-flask (Nunc) in 5% CO₂ incubator 37°C (Mettler, Germany).

Master mix preparation of bacterial suspension was incubated for 24 hours in 37°C before use in 25mL BHI broth or tryptose phosphate broth. 200mL of master mix bacterial suspension was then inoculated as the initial inoculated bacteria to each experimental tube-containing 4.8mL of ethanolic propolis in BHI broth or tryptose phosphate culture media and was measured as the initiation phase ($t_0 = 0$ hour) from all tubes. Bacterial growth rate analysis was performed by measuring the optical density or OD (for turbidity test) which was started after 24 hours incubation to several different ethanolic propolis concentrations in time-sequential manner using UV-mini 1240 spectrophotometer (Shimadzu) at 600 nm wave of length as described previously.^{12,13} Cytotoxic effect from the ethanolic propolis compound was observed by evaluating fibroblast cells viability using MTT-assay (at 550 nm wave of length) using 96-well plate with 2×10^4 seeded cells/well in 100 microliter culture media, and the absorbance detection was performed using ELISA reader plate (Bio-Rad, USA) which was conducted in the 3rd unit of LPPT UGM. For the cells viability calculation, the obtained-absorbances were normalized by the absorbance of the control cells (untreated cultured-cells — as 100% viability condition) times 100%. Statistical analysis was performed by SPSS 15.0 software.

Results

The effect of ethanolic propolis compound with different concentration to the bacterial growth inhibitory capacity could be seen in the figure 1 for *Enterococcus faecalis* and figure 2 for *Porphyromonas gingivalis*. Growth-curves in both figures showed us the alteration in bacteria growing pattern after applying ethanolic propolis treatment into each bacterial culture tubes, together with the control group as a normal baseline of bacterial growth pattern. Time sequential manner or time-course observation was performed for bacterial growing curve evaluation following the indicated ethanolic propolis concentration. The control group was treated with *aquades* sterile, while the untreated group was observed without any treatment. From the figure 1, we could observe that by increasing ethanolic propolis concentration, it reduced

Enterococcus faecalis growth capacity (showed as the lower level in exponential phase profile) in consistence with ethanolic propolis dose-dependent manner. In the figure 2, *Porphyromonas gingivalis* growth capacity also was decreased by increasing ethanolic propolis concentration. However, we could observe the growth inhibitory peak of ethanolic propolis effect in *Porphyromonas gingivalis* growth inhibition at around 0.4% of ethanolic propolis concentration.

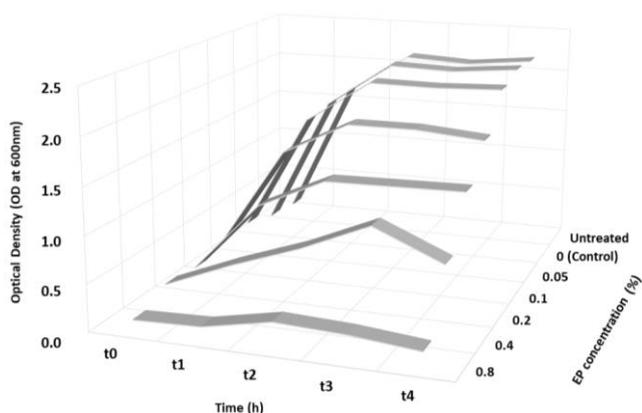


Figure 1. *Enterococcus faecalis* growth curve Optical density of bacterial growth (Turbidity test) in the time sequential manner at 0h (t₀), 4h (t₁), 24h (t₂), 28h (t₃), and 48h (t₄).

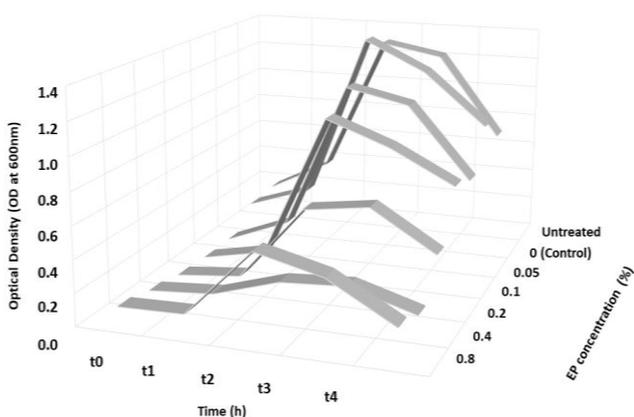


Figure 2. *Porphyromonas gingivalis* growth curve Optical density of bacterial growth (Turbidity test) in the time sequential manner at 0h (t₀), 6h (t₁), 24h (t₂), 30h (t₃), 48h (t₄).

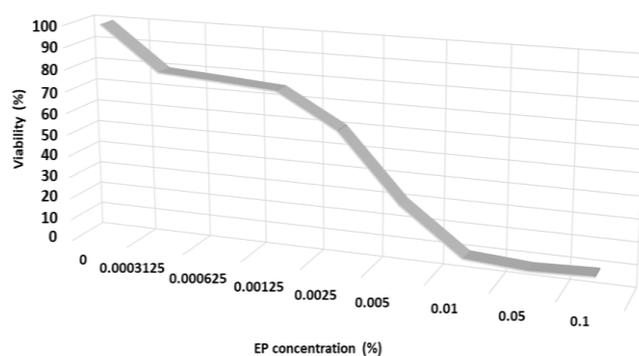


Figure 3. Fibroblast viability MTT-assay for fibroblast viability analysis (percentage of the living cells) along with the increasing ethanolic propolis (EP) concentration at 24 hours after seeding.

In order to evaluate the effectiveness of ethanolic propolis compound in bacterial growth inhibitory capacity, time-sequent of t₂ was determined as the *end-point* of exponential phase while t₀ was determined as the *initial-point* of bacterial growth profile. The optical density of t₂-t₀ representing the growing profile of bacteria which was used for effectiveness analysis. In the table 1, it showed the optical density decreasing value of *Enterococcus faecalis* significantly along with the increasing ethanolic propolis concentration p=0.000 by One Way Anova analysis with normal data distribution Sig. 0.094 by Shapiro-Wilk analysis. Similar result also could be observed in *Porphyromonas gingivalis* which was also decreasing in optical density significantly along with the increasing ethanolic propolis concentration p=0.009 by Kruskal-Wallis analysis with non-normal data distribution Sig. 0.002 by Shapiro-Wilk analysis. From the means OD_{t₂-t₀} of *Porphyromonas gingivalis* it showed to have only slight difference among the concentrations, while clear difference was observed at 0.4% ethanolic propolis concentration as the peak effect.

For further study related in the effect of ethanolic propolis compound to the host tissue, we utilized a yellow water-soluble tetrazolium dye (3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide) for being reduced enzymatically by mitochondrial *dehydrogenases* in a living cell to form formazan crystals which could be detected spectrophotometrically at 550nm. The result of this viability test in the figure 3 showed that fibroblast cells viability was

decreasing along with the increasing ethanolic propolis concentration.

Ethanolic propolis concentration (%)	OD <i>E. faecalis</i>	OD <i>P. gingivalis</i>
0	1.98	1.22
0.05	1.79	1.12
0.1	1.47	1.00
0.2	1.11	1.12
0.4	0.55	0.15*
0.8	0.17*	0.43

Table 1. Optical density of bacterial growth.

Since the data distribution of cells viability was normally distributed *Sig* 0.118 by Shapiro Wilk analysis, Pearson correlation analysis was then performed to confirm the relation between parametric bivariate variable ethanolic propolis concentration and cells viability. The results showed the Pearson product coefficient -0.673 with 0.023 of significance value which suggested there was a significant strong (*r* value: 0.60-0.79) negative correlation between cells viability and ethanolic propolis concentration.

Discussion

In our findings, ethanolic propolis compound exhibited an antimicrobial activity through bacterial growth inhibitory capacity which could inhibit bacterial growth rate on both *Enterococcus faecalis* and *Porphyromonas gingivalis* significantly in the dose-dependent manner (which the higher ethanolic propolis concentration showed the stronger bacterial growth inhibitory capacity effect). Interestingly, the dose-dependent inhibitory activity in *Porphyromonas gingivalis* was not as linear as in *Enterococcus faecalis* in along with the increasing ethanolic propolis concentration. Since the effect of ethanolic propolis compound had a peak effectiveness at 0.4% of concentration in *Porphyromonas gingivalis* inhibitory activity. This result is also supported by the previous study which utilized Iranian ethanolic propolis against gram-positive bacteria that shows high effectiveness in bacterial growth inhibitory capacity, while in gram-negative bacteria shows less effective effect which may

related to the existence of the outer membrane structure.¹⁴ This condition indicated that an effective concentration specificity of the active biological compounds was required in order to be functioned properly depending on a bacterial specific manner. Based on variance statistical analysis from the exponential phase of bacterial growth profile (t_2-t_0), our data significantly suggested the potency of ethanolic propolis in bacterial growth inhibitory activity which may related to the polyphenolic-derived component of propolis.¹⁵ Furthermore, this inhibitory activity result also indicated a broad-spectrum antibacterial capacity, not only in gram-positive but also gram-negative bacteria. This finding was in accordance with the previous report which also indicates the potency of ethanolic propolis solution as an alternative to chlorhexidine in antibacterial capacity for oral purposes.¹⁶ This alternative option has not been able yet for coming to the judgement since some other previous reports still shows the disagreements in method, dosage, formulation and the origin of propolis compound.¹⁷

Concerning on the biocompatibility issue, an adult human fibroblast cells were selected as an *in vitro* model which is closely related to the major cells population in the dental pulp, root canal, openly-wounded oral mucosa and *lamina propia* of the gingiva. MTT-assay was performed for cytotoxic analysis and the result indicated the opposite relation toward ethanolic concentration, in which the higher ethanolic concentration, the lower cells viability in return. Our data also indicated that even the less-effective ethanolic propolis concentration in both *Enterococcus faecalis* and *Porphyromonas gingivalis* bacterial inhibitory capacity (0.1% of concentration), it had already showed the decreasing cells viability (up to 9.48% in cells survival), which was classified as a strong toxicity effect to the cells viability (<30% cells survival).¹⁸ Our fibroblast cells viability data were resulted from a long term of ethanolic exposure time during the assay procedure. This duration of exposure may also induce the cytotoxic effect from ethanolic propolis to the host-cells. While local short exposure of propolis from *Apis mellifera* through irrigation procedure supports probing depth reduction.¹⁹ There were still controversial reports regarding propolis toxicity effect, since it was mainly reported only from *in vitro* studies while few were reported from *in vivo* studies.²⁰ As it may also

diverse among propolis components depending on the geographical factors, plant sources, and bee species¹⁵ which possibly lead to the variety of its biological property. Consideration for the selection and utilization of extraction solvent (method) and bulk-extraction compound (unpurified substances) also may contribute to the outcome which is not only in cytotoxic effect, but also antibacterial capacity. Different biological-compound preparation and substance-complexity have been reported for exhibiting significant different in biological properties, where the crude extract of a natural substance could be more effective and potential as an antibacterial agent compare to the purified substance. Furthermore, reducing substance complexity by performing fractionation is not always guarantee in reducing its cytotoxicity.²¹ Dynamic biocompatibility issues in the developing novel remedies which originally derived from the natural substances area challenge for the further investigations.

Conclusions

According to our findings, it could be concluded that both *Enterococcus faecalis* and *Porphyromonas gingivalis* growth abilities were significantly inhibited by the exposure of ethanolic propolis compound, particularly at 0.8% and 0.4% of ethanolic propolis concentration respectively. However, this ethanolic propolis compound at 0.1% has already highly toxic to the human fibroblast cells. Considering these findings, further investigations are required for overcoming biocompatibility problem to earn the optimum benefit from local propolis of *Apis Trigona* in developing oral antibacterial medicament.

Disclosure statement

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