ORIGINAL PAPER

EVALUATION OF BIO-COMPATIBILITY AND EFFECTIVENESS OF PROPOLIS *TETRAGONULA* SP. AS DENTAL ANTI-MICROBIAL AGENT

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ABSTRACT

Introduction: Bacterial invasion is an important concern in dental therapy procedures. Oral bacteria, such as *Enterococcus faecalis* (*E. faecalis*) and *Streptococcus mutans* (*S. mutans*) could be more virulent in dysbiosis condition. Utilization of anti-microbial agent, e.g., chlorhexidine digluconate (CHX), is commonly applied for bacterial control in dental practice, since it harbors significant bacterial eradication. However, bio-compatibility concerns arise along with chemical anti-microbial agent towards surrounding tissues. Propolis, with its' various bio-active compounds is reported to inhibit bacterial growth without artificial chemical substance, and making it as a promising alternative anti-microbial agent.

Objectives: This study aimed to evaluate anti-bacterial capacity of the propolis compound and its' cyto-toxic effects against human fibroblast.

Material and methods: 0.1% and 1% of both ethanolic- (EEP) and water-based (WEP) compounds of local propolis were prepared prior to experimental procedures. Both *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175 were measured at 625 nm for McFarland's initial adjustment, and turbidity at 600 nm for growth evaluation and anti-bacterial activity. Cyto-toxic effects of both extracts towards fibroblast cells were evaluated through cells viability test using MTS method. Shapiro-Wilk test was performed followed by ANOVA, *t*-test, or Mann-Whitney *U*-test.

Results: It showed that the EEP tended to be more toxic than the WEP against human fibroblast cells in higher concentrations. Both 1% of WEP and EEP showed significant bacterial inhibitory activities against *E. faecalis* and *S. mutans*.

Conclusions: These findings suggest the enhanced possibility of propolis-based compound as a promising dental anti-microbial agent.

Key words: *Enterococcus faecalis*, *Streptococcus mutans*, dysbiosis, water-based propolis, ethanolic-based propolis, bio-compatibility.

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INTRODUCTION

Pathogenic bacterial infection is one of the critical causes for tooth decay. Bacteria secretes various virulence factors, playing a role in damaging the structure of tooth. *Enterococcus faecalis* and *Streptococcus mutans* are involved in dental caries, periodontal tissue infections, and persistent infections in endodontic tissue [1, 2].

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In the attached biofilm, they strongly exhibit an acidic environment on the tooth surface, which in turn degrades in-organic structure, while proteolytic activities degrade the organic structure of tooth. Furthermore, they tend to be more protected from anti-bacterial agents in complexed or matured bio-film form [1, 3, 4]. It could also promote endodontic failure in the persistent bacterial dysbiosis.

Chlorhexidine digluconate (CHX) is commonly used as an anti-bacterial agent due to its' effectiveness and ability in reducing oral pathogenic bacteria [5, 6]. Several concentrations from 0.06% to 1% have been used in daily dental practice all over the world [5]. Unfortunately, in some reports, CHX is still harboring a concern due to its' cyto-toxicity effect towards surrounding oral tissues, even at 0.05% of CHX [7]. Its' cyto-toxicity effect occurs through the enhanced oxidative stress and intercellular Ca^{2+}, impairing mitochondrial function [8]. Therefore, other alternative anti-bacterial proprieties with less cyto-toxicity effects are always beneficial to be further investigated.

Recently, propolis has drawn attention due to its' health benefit effects. It belongs to natural material containing a mixture of resinous plant exudate with bees' secreted compounds, including wax and enzymes, which are naturally used to improve the hive's structure and to protect from outer threats [9, 10]. Propolis composition varies depending on several factors, such as bee species, surrounding plants, habitats, and seasons [11]. The main bio-active parts consist of phenolic and terpenoids [12, 13], and these substances have been widely reported to have anti-bacterial activity through affecting bacterial enzyme activities, hampering bacterial homeostasis, radicals scavenging activity, and reducing reactive oxygen species toxicity effect [14-16]. However, the variation in the exhibited biological effects are also correlated with the extraction and preparation procedures [17].

Ethanolic propolis extract (EEP) have been showed in many in-vitro studies to have an anti-bacterial agent and significant inhibitory capacity against oral bacteria [18, 19]. However, cyto-toxic effect of EEP on surrounding tissues and cells remains a concern. During conventional bio-active compound extraction procedures from its' raw material sources, several type of solvents could be applied; one of the alternative applicable solvents is water, which is assumed to be less toxic compared to the other solvents. The potential usage of propolis as an oral anti-bacterial with several possible preparation methods could be necessary to be performed in relation to bio-compatibility issues towards human tissues and cells.

An exploration and descriptive study on propolis compounds that originally come from Nglipar district, indicate the total of terpenes and phenolic compounds reaching 91.77% [20], showing anti-bacterial capacity [21, 22]. According to the best of our knowledge, no prior studies were found published on the anti-bacterial activity of water-based propolis extract (WEP) and cyto-toxic effect of the local propolis. Therefore, we would like to evaluate whether WEP from stingless bee *Tetragonula* sp. in Nglipar, Gunungkidul, Daerah Istimewa Yogyakarta, has a preferable anti-bacterial ability rather than EEP, without causing a pronounced cyto-toxic effect on the human cells. It was assumed that WEP may provide the similar anti-bacterial capacity with less cyto-toxic effect compared with EEP. This research could be considered as the first evaluation study formulating the preferable application of propolis-based medicament in both anti-bacterial and cyto-toxic effects. Hopefully, the results of this study would strengthen the existing knowledges about the usage of propolis-based material as a potential supporting medicine in oral bacterial control approaches.

OBJECTIVES

This study aimed to evaluate anti-bacterial capacity of the propolis compound and its' cyto-toxic effects against human fibroblast.

MATERIAL AND METHODS

ETHICAL CLEARANCE

The research protocol has met the feasibility according to the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences, UMY (No. 049/EC-EXEM-KEPK FKIK UMY/IV/2022).

MATERIALS

Raw materials of propolis were obtained from an apiary in Nglipar, Gunungkidul, Daerah Istimewa Yogyakarta. Isolates of bacteria *E. faecalis* ATCC (American type culture collection) 29212, *S. mutans* ATCC 25175, and human dermal fibroblasts-adult (HDFa) cell culture (Gibco C-013-5C, USA) were used, and technical laboratories and experimental procedures were supported by Molecular Medicine and Therapy laboratory of the Faculty of Medicine and Health Sciences (MMT FKIK) Universitas Muhammadiyah Yogyakarta.

PROPOLIS EXTRACT

Extract materials were prepared according to previous studies [19, 23], with some modifications in grinding and filtering process. Raw propolis was soaked in the liquid nitrogen and cut into smaller pieces, then grinded. Grinded propolis was stirred in 40% ethanol for EEP or distilled water for WEP for 48 hours at room temperature. The obtained propolis were then filtered using Whatman filter paper and evaporated in drying oven (Biobase, China) at 37°C. The extracted propolis was

dissolved in the brain heart infusion media (BHI, Oxoid, UK) or Dulbecco's modified eagle medium (DMEM, Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (FBS, Capricorn Scientific, Germany) for anti-bacterial or cyto-toxicity analysis, respectively. Mixtures were centrifuged at 10,000 rpm for 10 min at RT (Biocen, Ortho Alresa, Spain). Supernatants were filtered using a 0.22 µm filter (Himedia, India), and then diluted into a final concentration of 0.1% and 1%.

BACTERIAL COLONIES PREPARATION

McFarland's standard solution was prepared by dissolving 1% $BaCl_2$ in 1% H_2SO_4 into several McFarland scales: 0.5, 1, 2, 3, 4, and 5. The standard was measured at 625 nm, and the value was used to create a linear regression formula (R^2 = 0.999, y = 0.2626x – 0.01904). *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175 cultures were measured at the same wavelength as the initial standard. Optical densities (OD) were converted into McFarland's standard.

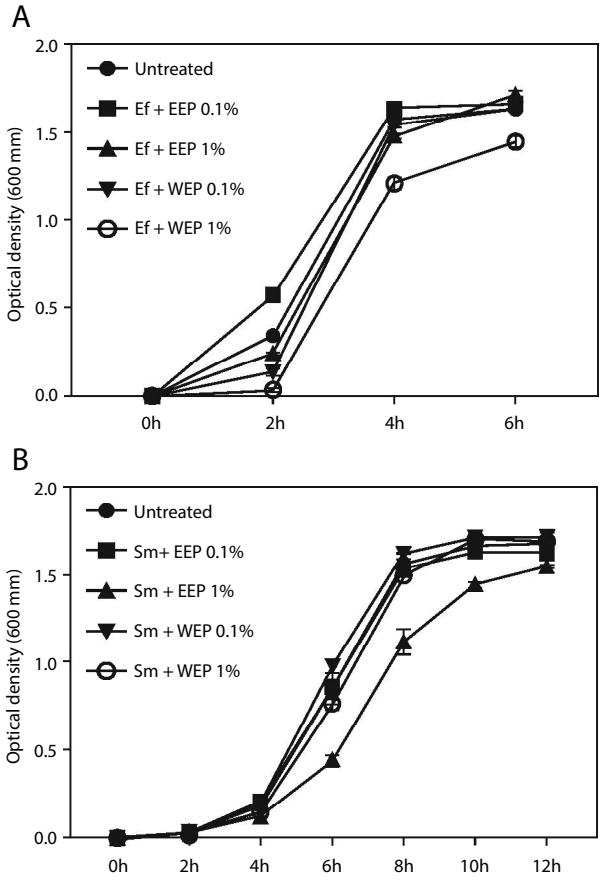

FIGURE 1. Growth curves of *Enterococcus faecalis* (Ef) (**A**) and *Streptococcus mutans* (Sm) (**B**), respectively, in EEP and WEP solutions. The OD at 600 nm was determined as bacterial turbidity. Each indicated points represented the mean value, while the SEM value was showed as an error bar on each graph

ANTI-BACTERIAL ACTIVITY

Both *E. faecalis* ATCC 29212 and *S. mutans* ATCC 25175 were cultured in BHI media and shaken overnight at 37°C. Bacterial cultures were then adjusted to 1 McFarland (equivalent to 3 x 10^8 colony forming unit (cfu/ml) at 625 nm (Halo RB-10, Dynamics, UK). Cultured bacterial suspension was added to culture media containing propolis for further 6 hours for *E. faecalis*, and 12 hours for *S. mutans* incubation at 37°C. Bacterial turbidity was re-evaluated every 2 hours at 600 nm.

CYTO-TOXICITY ANALYSIS (3-(4,5-DIMETHYLTHIAZOL-2-YL)-5-(3-CARBOXYMETHOXYPHENYL)-2-(4-SULFOPHENYL)-2H-TETRAZOLIUM-MTS METHOD)

HDFa cells were maintained in DMEM supplemented with 10% FBS and seeded in a 96-well culture plate until they reached around 75% cells confluency evenly. EEP or WEP was then added to the culture plate and incubated for 4 and 8 hours. After completing incubation time, the culture media was removed prior to CellTiter 96® aqueous one solution reagent (Promega, USA) incubation for 4 hours. The absorbances were measured at 490 nm (iMark™ microplate reader, Bio-Rad, USA).

STATISTICAL ANALYSIS

All data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, USA). Shapiro-Wilk normality test was then initially performed; if the data were normally distributed, ANOVA and Dunnet as a post-hoc or Student's *t*-test were further applied. Otherwise, it would be analyzed using Mann-Whitney *U*-test. Data were stated as mean ± standard error of the mean (SEM), with a significance level for each annotation: ns – non-significant; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^*p < 0.0001$.

RESULTS

ANTI-BACTERIAL ACTIVITY OF PROPOLIS EXTRACTS

Bacterial turbidities were measured at 600 nm every 2 hours, 6 hours for *E. faecalis* and 12 hours for *S. mutans*, to construct the growth curve of each bacterium. In Figure 1, the growth curve of each bacterium is demonstrated to have similar growth pattern along with the culture time.

We used two-time points at early and late exponential growth for anti-bacterial and cyto-toxicity tests. The bacteria cultures with similar initial cfu showed no significant difference at the early stationary phase (late exponential phase) (Figure 2); although different growth rates were observed during the exponential phase (Figure 1).

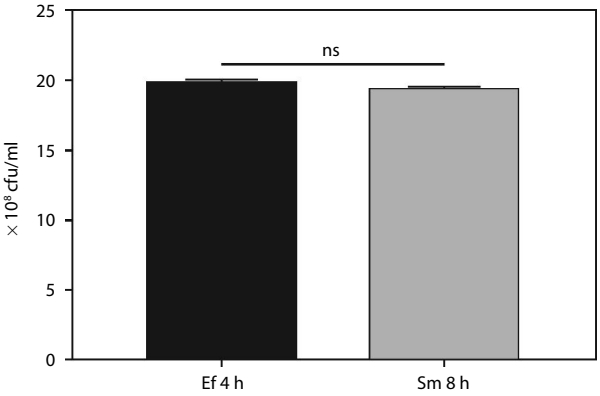

FIGURE 2. Number of bacterial colonies in late exponential phase. McFarland's scale values (at OD 625 nm) converted to cfu/ml (1 McFarland ≈ 3 x 10^8 cfu/ml). Ef – *Enterococcus faecalis*, Sm – *Streptococcus mutans*

After confirming the growth curves of the bacteria, OD values were used to determine the anti-bacterial capacity of EEP and WEP. 1% EEP showed inhibitory capability towards *E. faecalis* at early (2 hours) and late exponential (4 hours) phases (Figure 3A; $p < 0.0001$ and $p = 0.0005$, respectively). Similar result was shown in the 1% WEP solution ($p = 0.0134$ and $p < 0.0001$, respectively).

Furthermore, the anti-bacterial capacity of both EEP and WEP toward *S. mutans* was determined at the same phases as *E. faecalis*. Interestingly, in both 0.1% and 1% of EEP and WEP, no effect on *S. mutans* was shown at the early exponential phase (Figure 3B); however both 1% of EEP and WEP showed a significant anti-bacterial effect at the late exponential phase (Figure 3B; $p = 0.0019$ and $p = 0.0077$, respectively).

CYTO-TOXICITY EFFECT OF PROPOLIS EXTRACTS

For further application of safety concern, we conducted MTS analysis to evaluate the effect of both extracts on cells viability. The MTS procedure was carried out at 2-time points, i.e., 4 h and 8 h in accordance with bacterial growing profile. The difference in time points did not show any significant change. The results suggested that 1% EEP was responsible for having a significant cyto-toxicity towards fibroblast cells at 4 h and 8 h (Figure 3C; $p = 0.0014$ and $p < 0.0001$, respectively), with cells viabilities of 35.998 ± 2.773% and 67.371 ± 2.558%, respectively.

DISCUSSION

We used two-time points at early and late exponential growth for both anti-bacterial analyses. This consideration come from the preferred trend condition that several types of bacteria tend to exhibit and increase their virulence activity during the exponential growth phases [24, 25].

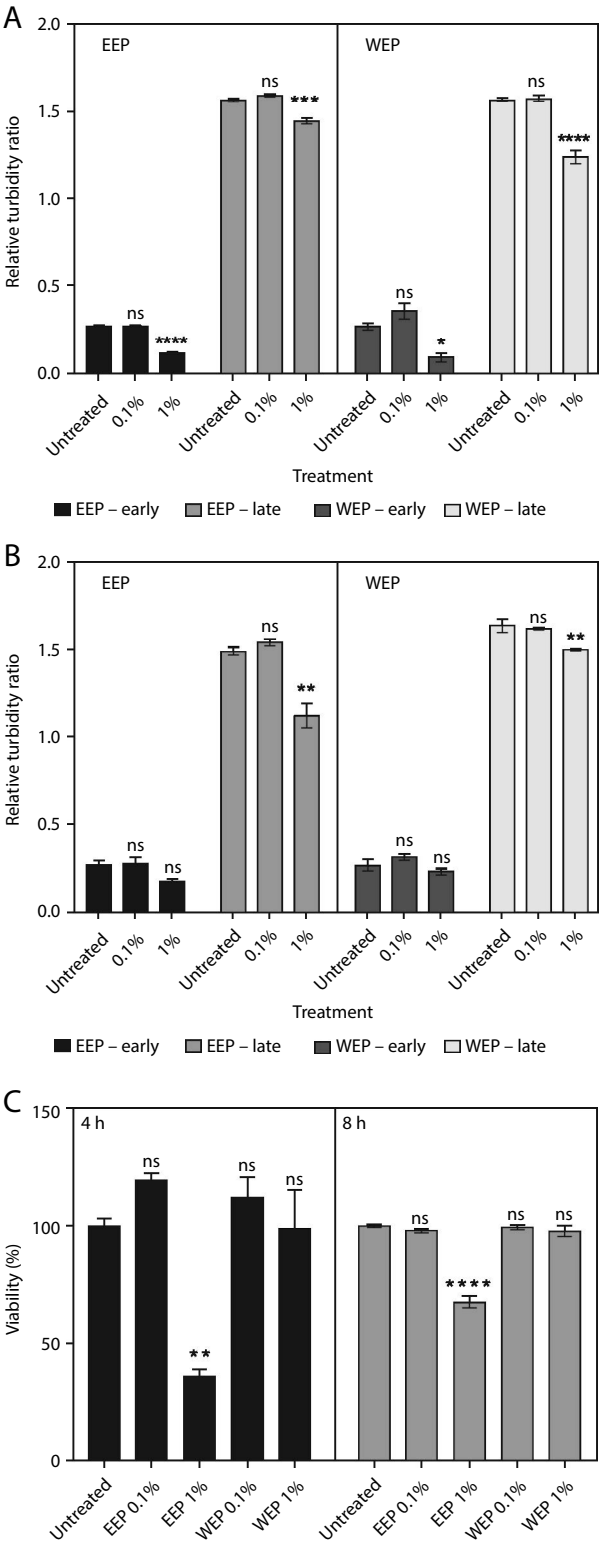

FIGURE 3. Bacterial turbidity of *Enterococcus faecalis* after 2 h and 4 h treatments (**A**), *Streptococcus mutans* after 4 h and 8 h treatments (**B**). Turbidity data represented the OD mean value of bacteria at 600 nm. Bio-compatibility of propolis extracts after 4 h and 8 h incubation (**C**). Feasible data indicated the absorbance values of each treatment's formazan reduction compound, which was compared with control absorbance (untreated group) value to obtain the viability percentage

In this study, we utilized *E. faecalis* and *S. mutans* as commonly known bacteria species in dental health problem. They were reported to produce antigen I/II, collagen-binding, and glucan-binding protein, which thought to have essential role in dental colonization ability [1, 26, 27]. The 0.1% concentration of the propolis extract was used, since it had already indicated bacterial inhibitory capacity in a previous study [19], whereas 1% utilization was a modified concentration from a study, in which it was assumed to show stronger anti-bacterial capacity [28].

The effect of both propolis extracts of EEP and WEP on *E. faecalis* seemed to be stronger than that of *S. mutans*. 1% propolis extract was able to inhibit *E. faecalis* since the early of the exponential phase, and still could be maintained until the late of exponential phase (Figure 3). However, different effectivity profile was observed towards *S. mutans*, where 1% EEP and WEP had expected effects in the late exponential phase (Figure 3). Therefore, under the same concentration as an ethanolic extract, water-based extract could provide similar effect. Moreover, our results exhibited similar pattern of anti-bacterial capacity against both *E. faecalis* and *S. mutans*, as supported by the previous studies that suggested anti-bacterial activity from both the extracts [29, 30]. Ethanolic propolis tends to have a stronger anti-bacterial capacity than water-based propolis, which could be assumed to be correlated with the higher ethanolic solvent concentration. The mixture of ethanol and water has wider range of polarity; thus, it can extract more type of compounds than sole water or ethanolic solution [31, 32]. Phenolic, terpenoid, and remaining compound of the propolis extract acted synergistically to disrupt the cell wall and membrane, leading to cellular leakage and death [33, 34]. However, the cyto-toxic effect along with higher ethanolic solvent has to be considered [35-37]. Therefore, these results suggested that both EEP and WEP might be used as potential anti-bacterial agents.

Cyto-toxicity evaluation showed that 1% EEP was observed to be toxic for fibroblast cells, regardless of the exposure duration. On the other hand, 0.1% EEP showed no cyto-toxic effect on the cells. Thus, according to ISO 10993-5:2009 [38], 1% of EEP was considered toxic because the cells viability percentage was below 70%. In the case of EEP, previous study also suggests similar tendency with our results in its' cyto-toxic effect, even with a lower EEP concentration. This difference, despite their similar tendency, could be affected by preparation procedures and some considerations within cells viability quantification procedures. In this study, we performed additional filtration using a 0.22 µm filter membrane for debris removal during propolis preparation to eliminate possible contaminant particles or other excess components within the extract. In a previous study [19], MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was utilized assay for the cells viability test, which can show different pattern to MTS due to the produced formazan in the MTS assay that could be more stabilized than MTT's formazan, suggesting sensitivity enhancement of the assay. Apart from the produced formazan stabilization, there are also some other possibilities for MTT assay in cellular toxicity involvement during the assay process due to the different tetrazolium compound's charges, which may affect the cellular membrane penetrability, and produce formazan crystal solubility problem, which can physically affect cellular damage [39-41]. Concerning bio-compatibility issue, it was interesting to see that WEP did not show any cyto-toxic effect towards fibroblast cells even in 1% propolis solution. This different cyto-toxic effect on the cells was speculated to be related to the solvent involvement. Some previous reported similar concern due to this solvent involvement since EEP may yield more phenolic compound compared with WEP [32]. Another study also showed that the excess of this phenolic compound confers cyto-toxicity towards normal cell [42].

Taken together, propolis extracts might be used as a supportive medicament for dental treatment according to previous studies, which reported the potential effectiveness of propolis in preventing disease advancement in in-vivo studies. The extracts could be applied as ointment or irrigant formulation [43, 44]. The supportive medicament or bacterial control agent should not only consider the effectiveness of anti-bacterial capacity, but also bio-compatibility properties towards surrounding tissues. According to our findings, small amount of propolis extract could exhibit significant inhibitory capacity towards bacterial growth activity by 1% concentration. This capacity was assumed to be related to active biological compounds of propolis, particularly phenolic [11, 31]. In this study, we found that 1% water-based extract can be used instead of ethanolic-based extract as a safer alternative option for propolis-based oral medicament, since it showed some effectiveness in oral bacteria without having significant cyto-toxicity effect on human cells.

This study suggest a new insight of local propolis potential for an effective anti-bacterial agent and tissue-friendly alternative oral medicament at the same time. There were some limitations within this study that could be related to the utilized bacteria, which did not directly represent the whole oral microbiome to be generalized in the actual clinical condition. Moreover, this study only covered the in-vitro perspective of propolis extracts bio-compatibility. Further studies are required for investigate responsible substances of the extracts, their mechanism in oral pathogenic bacteria, including the in-vivo bio-compatibility approval and clinical evaluation for acquiring clinically applicable formulation of propolis extract.

CONCLUSIONS

Based on the results of the present study, ethanol-based propolis extract (EEP) was supportively effective in hampering oral pathogens growing capacity, but

had a pronounced cyto-toxic effect. On the other hand, water-based extract (WEP) of propolis provided similar anti-bacterial capacity, with no cyto-toxic effects observed. Therefore, it is considered that WEP can be utilized as a safe supportive oral anti-bacterial medicament instead of EEP.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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