The Antimicrobial Properties of Malaysian Propolis as Intracanal Medicament in Endodontics

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Abstract: Numerous studies have shown that propolis, from the sting and stingless bees, possessed antimicrobial, antifungal, antidiabetic, antitumoral and antioxidant properties. Propolis produced by stingless bees, *Heterotrigona itama*, also possessed these properties but in varying intensity. Exploiting this natural product with antimicrobial properties against *Enterococcus faecalis* (EF), would be advantageous in endodontics. Particularly where the current synthetic medicament, calcium hydroxide ($Ca(OH)_2$), failed to remove these bacteria, predominantly found in failed root canal-treated (RCT) teeth. The aims of this study are to determine the antimicrobial properties of Malaysian propolis (MP) against *Enterococcus faecalis* and to compare the antibacterial effect of MP with $Ca(OH)_2$ as an intra-canal medicament. Raw propolis was purchased from Humaira Honey Sdn Bhd, Lenggong, Perak and the ethanolic extraction method was carried out until lyophilization. Antimicrobial susceptibility testing (AST) was done on five samples, namely, MP, $Ca(OH)_2$, 2% chlorhexidine (CHX), 70% ethanol, and sterile normal saline (SDW), where CHX, 70% ethanol and SDW as controls. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) methods were carried out. The MIC and MBC for MP and CHX were done using the micro-dilution method on 96-well, while $Ca(OH)_2$ were performed using the serial macro-dilution method. The results were analysed by Kruskal Wallis test with Mann-Whitney posthoc test and repeated-measures ANOVA with Bonferroni post hoc test ($p < 0.05$). The mean MIC from three trials that were carried out on the samples was CHX (<0.008%) < MP (0.03%) < $Ca(OH)_2$ (0.62%). While the MBC values for three trials were found to be CHX (0.011%) < MP (0.07%) < $Ca(OH)_2$ (1.25%). In conclusion, MP was shown to be antibacterial and effective against *Enterococcus faecalis* and is more effective when compared to $Ca(OH)_2$, which is commonly used as an intra-canal medicament. Further research is needed to test MP's clinical efficacy.

Keywords: Malaysian propolis, Calcium hydroxide ($Ca(OH)_2$), Intra-canal medicament, Antibacterial, *Enterococcus faecalis*.

INTRODUCTION

Propolis, or bee glue, Is a compound produced by the sting and stingless bees, are well known for their medicinal properties, which have many precious salubrious properties (Więckiewicz *et al*., 2013). Six hundred stingless bee species belong to the Meliponini tribe in the Apidae family, which are found in the highest number in South/Central America, tropical Africa, Southeast Asia and Australia (Hrncir *et al*., 2016). Chemical research on Meliponini propolis has resulted in the discovery of numerous new molecules, including antibacterial ones (Popova *et al*., 2019).

In Malaysia, propolis from both sting and stingless bees are readily available, with studies of stingless bee propolis on the rise. A recent study on Malaysian propolis from 4 species, namely, *Heterotrigona itama* (HI), *Geniotrigona thoracica* (GT), *Lepidotrigona terminate* (LT), and *Tretrigona apicalis* (TA), showed resulted in HI producing the highest antioxidant activity compared to the other 3 species (Mat Nafi *et al*., 2019). Ibrahim *et al.,* also stated that the methanol extract of Malaysian propolis produced by two commonly found stingless bees, *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT), works better in inhibiting the growth of *Staphylococcus aureus* better than Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria.

But the propolis component depends on where it is collected (Koru *et al*., 2007). It possesses several biological activities such as anti-inflammatory, immunostimulatory, antiviral, and antibacterial (Russo *et al*., 2002). It also contains many plant phenolic compounds, of which flavonoids occupy about half of the components. It is bactericidal, which kills bacteria by inhibiting their motility, via the inhibition of RNA-polymerase, disorganization of cytoplasm, cytoplasmic membrane and cell wall, and suppression of protein synthesis (Mirzoeva *et al*., 1997; Pepeljnjak & Kosalec, 2004).

Root canal therapy is needed to ensure the dental infection does not spread to surrounding tissues,

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including the jaw bones. Intra-canal medicament placement is a standard procedure in root canal treatment, and its antimicrobial property is a suitable disinfectant to be applied in-between visits. Calcium hydroxide ($Ca(OH)_2$) is the most common intra-canal medicament. However, it is synthetically produced, and the efficacy of the antimicrobial effect is low. Its action mechanism neutralizes the intracanal pH due to its high alkalinity, pH 12.5-12.8 (Mohammadi & Dummer, 2011). This results in ionic dissociation of Ca^{2+} and OH^- ions which induces hard tissue deposition. The bactericidal mechanism of action of $Ca(OH)_2$ is protein denaturation and damage to both DNA and cytoplasmic membranes (Mohammadi & Dummer, 2011). However, a study showed that $Ca(OH)_2$ might be neutralized due to the buffering action of the deepest layer of dentinal tubules adjacent to dentino-enamel junction causing microorganisms to survive (Madhubala *et al*., 2011)

Thus, a safer natural substitute that will still offer similar benefits to $Ca(OH)_2$ needs to be researched. So, propolis is suggested since there are numerous studies conducted on it. In root canal retreatment procedures, when primary root canal treatment fails, it offers a viable treatment alternative to retrograde endodontic surgery. The failure of primary root canal treatment may be due to the presence of missed canals, survival of bacteria within the canal, or in cracked teeth. *Enterococcus faecalis* is the most common bacteria in failed root canal treatment (Zhang *et al*., 2015). It is a Gram-positive coccus, a facultative anaerobe that occurs singly, in pairs or short chains (Stuart *et al*., 2006). It can resist intra-canal medicament such as $Ca(OH)_2$ by maintaining pH homeostasis. Studies on Malaysian propolis produced by HI against *Enterococcus faecalis* still need further research.

The aims of this study are to determine the antimicrobial properties of Malaysian Propolis against *Enterococcus faecalis* and to compare the antibacterial effect of MP with $Ca(OH)_2$ as an intra-canal medicament.

MATERIALS AND METHODS

Extraction of Propolis

Propolis was extracted following the methods of ethanolic extract of the three studies but with some modifications Al-Bayati *et al*., 2017; Ismail *et al*., 2020; Al-Masoodi *et al*., 2022.

Raw propolis produced by *Heterotrigona itama* bee was purchased from Humaira Honey Sdn Bhd, Lenggong, Perak. Five grams of raw propolis was weighed and stored in a deep freezer (-80˚C). The propolis was minced by using a small dry blender. Then, 100ml of 70% ethanol was used to extract the active ingredients of propolis. Both substances were mixed in a 200ml conical flask. The mouth of the conical flask was covered by parafilm, followed by covering the entire flask with aluminium foil. Sonication of the mixture was done using an ultrasonic bath for 30 minutes under 27˚C at a moderate rate. The mixture was filtered by Whatman paper no.1 and left overnight. Filtrate was evaporated using a rotary evaporator to evaporate the ethanol for 2 hours until appropriate viscosity was obtained. The vaporized filtrate was transferred into a universal bottle and slanted 45˚ to increase the total surface area of the filtrate. The bottle was stored inside a deep freezer (-80˚C) overnight. The filtrate was lyophilized for 2 days to dry the sample, and the final product was stored for further use in a -20˚C chiller. Figure **1** shows the extracted Malaysian Propolis in powder form.

Figure 1: Extracted Malaysian propolis after lyophilization (in powder form).

Preparation of Bacteria

Bacteria were prepared following methods described by Ismail *et al*. (2020) with some modifications. *Enterococcus faecalis* (ATCC 29212) were rehydrated with 0.5ml of Brain-Heart Infusion Broth (BHIB). Fifty percent Glycerol was placed in 5 different cryovial tubes, and 0.1ml of inoculated BHIB was pipetted to each cryovial tube, respectively. Four of the cryovial tubes were stored as stock culture. The remaining cryovial tube was used to subculture the bacteria on blood agar and left for incubation overnight at 37˚C.

After one day of incubation, an assessment of bacterial growth was done. Non-haemolytic, circular

and convex colonization was noted, showing positive results on the growth of bacteria. Another 5 stocks culture of *Enterococcus faecalis* was prepared from the grown bacteria on the blood agar. The stock culture was prepared by pipetting 0.5ml of brain-heart infusion broth into cryovial tubes and added with 0.5ml of 50% glycerol. Ten microliter of *Enterococcus faecalis* was brewed to the cryovial tubes. All the cryovial tubes were stored in a deep freezer (-80˚C). Figure **2** shows the colony of *Enterococcus faecalis* grown on blood agar.

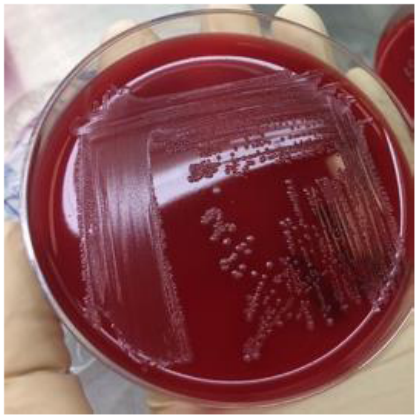

Figure 2: Freshly grown *Enterococcus faecalis*.

Preparation of McFarland Standard

McFarland Standards is used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the test suspension with that of the McFarland Standard. A fresh, pure culture of *Enterococcus faecalis* was inoculated in the blood agar before this procedure. A volumetric micropipette added approximately 3ml of sodium chloride (NaCl) to the test tube. *Enterococcus faecalis* was streaked using a sterilized loop and mixed into the sodium chloride (NaCl) solution. The solution was stirred and shaken until the solution appeared homogenous and free of clumps. The turbidity of (0.5) was measured using a densitometer.

All freshly grown bacterial suspensions in 3ml of sodium chloride (NaCl) were suspended to 1.5 x 10^8 CFU (colony forming unit)/mL according to the turbidity of 0.5 McFarland test standard.

Antibacterial Assessment - Antibiotic Susceptibility Testing (AST)

AST is widely recognised as a cornerstone of the management of infectious diseases. Despite the shortcomings of the traditional AST methods, their use is required in clinical practice to obtain the correct results (Gajic *et al*., 2022). The AST using the well-dilution method outlined below was based on a study by Ismail *et al*. (2020), with some modifications (Ismail *et al*., 2020). AST is a test to determine the antimicrobial properties of a sample. It was done for all the samples, including the MP, $Ca(OH)_2$, CHX, 70% ethanol, and sterile normal saline. A zone of inhibition surrounded the well containing Malaysian Propolis, 2% chlorhexidine, and $Ca(OH)_2$ against *Enterococcus faecalis*.

Three Mueller Hinton Agar (MHA) were prepared and divided into five portions labelled as Malaysian propolis, calcium hydroxide, 2% chlorhexidine, 70% ethanol, and sterile normal saline, respectively. A sterile cotton swab was dipped into the suspension of *Enterococcus faecalis*. While removing the swab from the tube, the soaked swab was rotated firmly against the upper inside wall of the tube to get rid of excess fluid. The cotton swab containing the culture was swabbed evenly onto the MHA plate. Five wells were prepared using a cork-borer size of 6 mm to deposit the samples, including Malaysian propolis, calcium hydroxide, 2% chlorhexidine, 70% ethanol, and sterile normal saline, respectively. 2% chlorhexidine is a positive control, while sterile normal saline is a negative control in this research. Approximately, 50ul of samples was added to their respective well using a volumetric micropipette. All the previous steps were carried out for the second and third plates. All three MHA plates were incubated at 37˚C and observed after 18-24 hours.

Minimum Inhibitory Concentration (MIC) test and Minimum Bactericidal Concentration (MBC) test

Preparation of *Enterococcus faecalis* with a McFarland standard of 0.5 is done. The bacteria were diluted to 10^8 CFU/ml using sterile normal saline. The Minimum Inhibitory Concentration (MIC) values for the *Enterococcus faecalis* were defined as the lowest concentration of Malaysian Propolis, 2% chlorhexidine (CHX), and $Ca(OH)_2$, which inhibited the visible growth of microorganisms.

Using the same MP and CHX concentrations, the MIC test was analysed by broth micro-dilution method. Ninety-six-well microplates were used in the broth microdilution method for MP and CHX. While for $Ca(OH)_2$, the MIC was carried out using the serial dilution method in a test tube. Twelve wells were allocated and labelled 1 to 9, 10 and 11 as the positive and negative control, respectively, and 12 as the sterility check. This method suspended 50ul of Mueller

Hinton broth into all microplate wells. The dilution of 100ul of MP and CHX was suspended into microplate wells no 1 and no 10. With a sterile micropipette and tips, 50ul was transferred from well no 1 to well no 2 after thorough mixing. This procedure was repeated until well no 9. From well no 9, 50ul was discarded. Subsequently, the 5ul suspension of *Enterococcus faecalis* was added to microplate wells containing 100ul for well no 1 and 50ul for well no. 2 to no. 9 and no 11(negative control). Well no 10 served as the positive control as it contained broth 100% of MP, while no 11 served as the negative control, which contained only broth and bacteria. The procedures were repeated for CHX. While for $Ca(OH)_2$, 12 test tubes were allocated, and the procedures were repeated as same as MP, but in a ratio of 1:20. To determine the MIC values, the incubations were carried out at 37°C for 24 hours. The MIC value was determined by examining the change or turbidity of each well. The wells examined for visible growth were recorded as (+) and no growth as (-).

The MBC of *Enterococcus faecalis* was determined by subculturing all the content from the well and test tube onto the MH agar plate. The concentration of the sample that showed no visible growth of *Enterococcus faecalis* was the MBC.

RESULTS

Antibiotic Sensitivity Test (AST)

After a 24-hour incubation at 37˚C, the samples were uncovered in an aseptic environment and were observed. The diameter of inhibition zones was calculated using Vernier calliper, with results shown in Figure **3**.

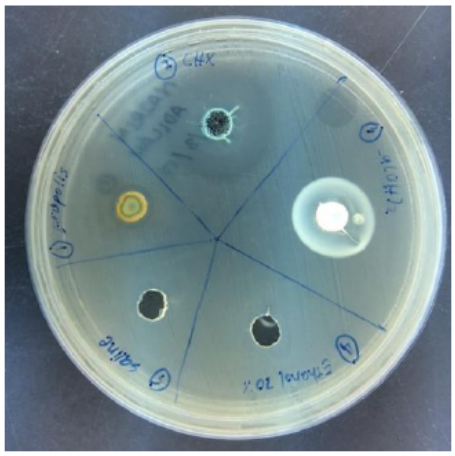

Figure 3: Results in triplicate for Antimicrobial Sensitivity Testing on: MP, Ca(OH)2, 2%CHX, 70% ethanol, and sterile normal saline.

Minimum Inhibitory Concentration (MIC) Test and Minimum Bactericidal Concentration (MBC) Test

The MIC values for all the samples took much work to be determined visually as the turbidity changes between each well were apparent. Thus, the MIC values was determined after the MBC test. All the mixture from each well and test tube in the MIC test were cultured into an MH agar. The growth of the bacteria was examined after 18-24 hours.

Table **1** below shows the mean resulting growth of *Enterococcus faecalis* in the MIC and MBC test for MP, $Ca(OH)_2$, and CHX:

The mean MIC for Malaysian propolis was 0.03% (0.32mg/ml), 2% chlorhexidine was <0.008% and calcium hydroxide was 0.62% (6.25mg/ml). 2% chlorhexidine showed the highest potency, followed by Malaysian Propolis and calcium hydroxide. This result showed that Malaysian Propolis required a much lower concentration for inhibitory activity against *Enterococcus faecalis* than calcium hydroxide.

DATA ANALYSIS

Statistical Package for the Social Science (SPSS v24) was used to analyse the data. They were analysed using the Kruskal Wallis test with Mann-Whitney post hoc test and repeated-measures ANOVA with Bonferroni post hoc test ($p < 0.05$).

DISCUSSION

The success rate of endodontic treatment is fairly predictable, with 86-98% success. However, the needs to be more consensus in the literature about guaranteeing success in every endodontic treatment. Failure of endodontic treatment can be defined in many ways. Some said that the recurrence of clinical symptoms with peri-apical lesions or radiolucency is a sign of failure in endodontic treatment. Various factors can be attributed to endodontic failures, such as the persistence of bacteria inside the canal, inadequate obturation of the canal, overextensions of root-filling materials and coronal leakage. Treatment for failure endodontic cases is usually to do retreatment of the tooth. Thus this retreatment will jeopardize the success rate of the treated tooth. Most of the intra-canal medicaments Used in current practice are low in antibacterial properties. The result revealed that CHX had the most effective antimicrobial properties compared to MP and $Ca(OH)_2$. Despite chlorhexidine

Table 1: The Mean MIC and MBC for the Three Trials of Samples Against *Enterococcus faecalis*

Trials	Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	%	mg/ml	%	mg/ml
MP 1	0.02	0.19	0.04	0.39
MP 2	0.04	0.39	0.08	0.78
MP 3	0.04	0.39	0.08	0.78
Mean	**0.03**	**0.32**	**0.07**	**0.65**
$Ca(OH)_2$ 1	1.25	12.5	2.5	25
$Ca(OH)_2$ 2	0.31	3.125	0.63	6.25
$Ca(OH)_2$ 3	0.31	3.125	0.63	6.25
Mean	**0.62**	**6.25**	**1.25**	**12.5**
CHX 1	0.008	0.078	0.016	0.156
CHX 2	<0.008	<0.078	0.008	0.078
CHX 3	<0.008	<0.078	0.008	0.078
Mean	**<0.008**		**0.011**	**0.104**

having the lowest concentration inhibiting *Enterococcus faecalis* compared to propolis and calcium hydroxide, a study by (Chang *et al*., 2001) found that chlorhexidine was extremely cytotoxic to periodontal ligament (PDL) cells. The mechanism inhibits double-stranded nucleic acid content, protein synthesis, and mitochondrial activity. They also stated that the toxic antimicrobial properties of chlorhexidine used in root canals may impede periapical healing.

Propolis, which possesses antibacterial, anti-inflammatory, and antioxidant properties, has been used widely since ancient times to treat many diseases. The active compounds from propolis, such as flavonoids, CAPE, and flavones can be obtained by propolis extraction using ethanol. Propolis can be extracted using sonication, traditional maceration, and microwave-assisted methods. A study by (Trusheva *et al*., 2007) found that extraction using the maceration method required longer time frames and low extraction yield. While using microwave-assisted extraction, the yield was rapid, but it contained a large amount of non-phenolic and non-flavonoid materials. The ultrasonic extraction method was chosen in this research as it provides a high extraction yield of phenolic compound and requires less time frame. The ratio of propolis to organic solvents is 1:20. According to (Sun *et al*., 2015), extraction of propolis using not more than 75% ethanol gives the maximum extraction yield of the phenolic compound from the propolis. Extraction using more than 75% ethanol will reduce the total phenolic compound and total flavonoid compound in extraction yield. In this research, 70% ethanol serves as an organic solvent. This is to make the extracted propolis soluble in water for dilution and a more active compound being extracted. Since the first objective of this research is to determine the antibacterial effect of Malaysian propolis, extraction of propolis using the sonication method with 70% ethanol that acts as solvent is preferable.

The study's findings showed that MP significantly inhibited the growth of *Enterococcus faecalis* bacteria. Compared to $Ca(OH)_2$, MP's mean MIC was 0.62%. In addition, MP (0.07%) had lower MBC values than $Ca(OH)_2$ (1.25%). These results imply that MP is superior to $Ca(OH)_2$, the current intra-canal medication, preventing the development and eliminating *Enterococcus faecalis*.

The results from this study are encouraging since they demonstrated MP's potential as a substitute antibacterial agent for endodontic treatments. The ineffectiveness of $Ca(OH)_2$ in completely removing *Enterococcus faecalis* from root canal treated teeth emphasises the need for alternate therapies. MP is a strong candidate for additional research and inclusion in clinical practice due to its inherent antibacterial characteristics.

Clarifying the precise bioactive substances in MP responsible for their antimicrobial properties could be the subject of future research. Finding these substances would help with the development of specialised antimicrobial medicines as well as a better understanding of the mechanisms underlying MP's antibacterial activity.

It is also essential to note that the propolis of different origins may affect the composition and its antimicrobial properties. Many studies have shown that that propolis has antibacterial properties. In this particular research, we tested the Malaysian Propolis against *Enterococcus faecalis.* The extracted Malaysian propolis showed antibacterial properties against *Enterococcus faecalis*. The MIC value of the extracted Malaysian propolis against *Enterococcus faecalis* was 0.03% (0.32mg/ml).

LIMITATION

Issues Regarding Calcium Hydroxide($Ca(OH)_2$)

The natural colour of calcium hydroxide, which is white, affects the turbidity of the micro-dilution. Hence was challenging to assess by the naked eye to determine the MIC value. Thus, in this study, the dilution was sub-culture on Mueller-Hinton agar to observe the bacterial growth.

Apart from that, the paste-like calcium hydroxide will have the tendency to harden and clog. It was difficult to manipulate the $Ca(OH)_2$ as it dried upon pipetting using a volumetric micropipette. Nevertheless, we piped it by dilution with sterile normal saline with a ratio of 1:5 ($Ca(OH)_2$: sterile normal saline).

During the antimicrobial assessment of $Ca(OH)_2$ against *Enterococcus faecalis*, the zone of inhibition was challenging to be determined since there was the presence of white coloured layer covering the inhibition zone.

Issues Regarding the Extraction of Malaysian Propolis

To maintain the standard of research procedures, mechanical power such as a rotary evaporator and freeze-dryer machince to extract propolis were needed in this study. However, some circumstances in any research could not be avoided, such as the freeze-dry machine has gone haywire. For this research, we decided to proceed without the lyophilisation process.

Moreover, due to limited sources in the state of Selangor, we needed more sources of Malaysian propolis in a short amount of time hence it will delay the research timeline.

In addition, if we were to get more sources in one particular place, it would compromise the environment of *Heterotrigona itama* sp.

Future research recommendation is to focus on elucidating the effectiveness of Malaysian Propolis by using animal models as well as conducting comprehensive clinical studies.

CONCLUSION

MP was found to be antibacterial and effective against *Enterococcus faecalis* and it was also more effective when compared to $Ca(OH)_2$, which is commonly used as an intra-canal medicament. Further research is needed to test MP's clinical efficacy.

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CONFLICT OF INTEREST

The authors declared there were no conflict of interest in conducting this study.

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