

Comparing Antimicrobial Effects of *Heterotrigona itama* Propolis from Two Regions of Malaysia Against *Enterococcus faecalis*

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Abstract: *Objective:* This present study aimed to determine and compare the antimicrobial properties of Malaysian propolis (MP) produced by *Heterotrigona itama* from different regions of Malaysia, against *E. faecalis*.

Methods: The propolis were obtained in Lenggong MLP (North Peninsula Malaysia) and Raub MRP - (Central Peninsula Malaysia). The antibacterial activities of the ethanolic extracts of propolis were conducted using antimicrobial susceptibility test (AST), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The effect of these extracts on the cell morphology were observed using scanning electron microscopy (SEM). Calcium hydroxide and 5% dimethyl sulfoxide (DMSO) were used as controls.

Results: Statistical analysis was done using unpaired t-test to differentiate the difference of the two averages. The ethanol extracts of MLP showed higher activity compared to MRP at 50mg/ml on *E. faecalis* with zone of inhibition of 14.33mm and 10.00mm respectively. MIC test for MRP and MLP was 0.78125mg/ml and 3.125mg/ml respectively. Whereas the MBC value for both MRP and MLP was 12.50mg/ml and 50.00mg/ml respectively. SEM analysis showed that MP disrupted the size, cell morphology and the structure of the bacteria cell wall.

Conclusion: Our study showed MRP outperformed MLP against *E. faecalis* via MIC, MBC and SEM. Dissimilarities of surrounding trees in Raub and Lenggong may provide the variable chemical source contributing to this contrast. Thus, these stingless bees have a very special relationship with plant resins, relying on them for the very survival of their nests.

Keywords: Antimicrobial, *Enterococcus faecalis*, *Heterotrigona itama*, Intracanal medicament, Malaysian propolis.

INTRODUCTION

Failure of endodontic treatment can be caused by various factors, including improperly cleaned and obturated root canals, leaky coronal seals, and it is majorly caused by the bacterial persistence (Alghamdi & Shakir, 2020). Endodontic infections are polymicrobial and *Enterococcus faecalis* is one of the most frequent microorganisms implicated in failure of endodontic treatment (Barbosa-Ribeiro *et al.*, 2016). In the human oral cavity, patients with post treatment apical periodontitis or refractory marginal periodontitis have frequently been identified with predominance of *E. faecalis* due to their resilience and possession of virulence factors (Pinheiro & Mayer, 2014). Data collected from several recent research shows that the prevalence of this Gram positive, facultative anaerobic coccus, in failed endodontic cases, was more than 75% via polymerase chain reaction as a detection method (Hargreaves *et al.*, 2016).

It can survive in harsh conditions, e.g., high salt concentration, alkaline environment (pH > 11.5) and temperature levels >45°C (Alghamdi & Shakir, 2020; Pinheiro & Mayer, 2014). Indeed, previous study reported that it is resistant against calcium hydroxide [Ca(OH)₂] which is normally used for intracanal dressing (Pimenta *et al.*, 2015). The same study also stated that it is difficult to eradicate this microorganism even after biomechanical treatment due to its ability to penetrate and colonize deeply within the dentinal tubules, by binding to collagen (Pimenta *et al.*, 2015). Therefore, the process to remove and clean the root canal using mechanical and chemical means become complicated (Rodríguez-Niklitschek & Oporto V, 2015). Due to these undesirable properties, it is able to resist endodontic medicaments in various ecological conditions to cope well with unfavourable conditions inside the obturated canal (Pinheiro & Mayer, 2014).

The most commonly used intracanal medicament is Ca(OH)₂, because of its antimicrobial properties, osteogenic potential and promoting of a temporary physical sealing of the root canal (Pimenta *et al.*, 2015). The number of microorganisms present in

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endodontic infections is restricted by the antimicrobial properties of Ca(OH)_2 , along with low oxygen levels. However, *E. faecalis* can survive during nutrient deficiency and withstand or suppress the action of antimicrobial agents used during endodontic medicaments environment which exist in cleaned and filled canals (Rodríguez-Niklitschek & Oporto V, 2015). Several justifications have been provided regarding *E. faecalis*' ability to withstand Ca(OH)_2 in the root canal. Raising the pH to at least 11.5 is crucial in order to stop the growth of *E. faecalis*, and Ca(OH)_2 appears to be the ideal substance because of its alkaline pH range of 12.5 to 12.8 (Dohyun & Euseong, 2014). However, this pH brought by the Ca(OH)_2 is very unlikely to be achieved in the root canal due to the dentin's buffering capacity that neutralize the alkaline environment (John *et al.*, 2015). Furthermore, *E. faecalis* can regulate the pH homeostasis within its cell and the environment by having cytoplasm's buffering capacity and proton pump that translocate the hydrogen ions into the cells by means of maintaining internal pH (John *et al.*, 2015). The significant flaws in the current intracanal medications to eradicate microbes on post-endodontic treatment has led to studies that aimed to find alternative substances from natural products, with stingless bee propolis being one of the best options (John *et al.*, 2015; Shabbir *et al.*, 2020). Propolis from stingless bee has been shown through research to have good antibacterial quality and potency that is beneficial for medical and therapeutic purposes. However, the antibacterial properties of stingless bee propolis and its effect towards pathogens have received little attention in current research (Ismail *et al.*, 2021).

Propolis is commonly known as "bee glue", which is a generic name for a resinous substance from different plant types accumulated by the bees (Ibrahim *et al.*, 2016a). Bees collect plant leaves, flower buds, pollen, plant resins and fill their hives with exudates. While transporting their "trophies" back to home, their own secretions like wax and saliva are mixed with these plant compounds, causing complex reactions which lead to propolis formation (Yuan *et al.*, 2020). Propolis serves in sealing holes and cracks as well as the reconstruction of the beehive (Pasupuleti *et al.*, 2017). It is also used to smooth the inner surface of the beehive, maintain the internal temperature of the hive (35°C) and prevent weathering and invasion by predators (Pasupuleti *et al.*, 2017). More than 300 components have been identified, including organic substances such as phenolic compounds, esters and many different known flavonoids and flavanones

(Pimenta *et al.*, 2015). In addition, an increasing number of studies have shown that biological activity of propolis is closely linked to this chemical complexity (Yuan *et al.*, 2020). However, the composition of propolis depends on the regional climate, type of plants that is accessible to the bees, and time of year it is collected (Ibrahim *et al.*, 2016a). Yuan *et al.*, 2020 has shown that there are relevant differences in the chemical composition between different types of propolis.

Studies have been reported that propolis from various geographical region has a wide range of biological activities such as antiseptic, anti-inflammatory, antioxidant, antibacterial, anti-fungal, antiulcer, anti-cancer, and immunomodulatory properties (Ibrahim *et al.*, 2016). When compared to other honeybee species, research indicates that the propolis produced by the stingless bee (lebah kelulut) species is more potent in term of its biological properties (Ibrahim *et al.*, 2016; Salleh *et al.*, 2022). As a way to compensate their lack of defence ability, stingless bees produced greater propolis, both in terms of quality and quantity. It will serve as a temperature regulator, a sealant to repair hive damage, and a disinfectant to keep the bee colony free of pests and microorganisms because of its antimicrobial properties (Salleh *et al.*, 2022). Stingless bee propolis play a part as an antibacterial agent over many bacterial infections due to its ability to inhibit the growth of various bacteria (Mat Nafi *et al.*, 2019). The extracts have various pharmacological uses in treating different diseases due to the diversity of its chemical composition (Pasupuleti *et al.*, 2017). It has long been utilised in traditional medicine due to the wide range of bioactivities attributed to propolis (Teerasriprecha *et al.*, 2012). Geographical locations where the source plants may vary relative to the local flora of the site collection and seasons have been observed affect the chemical composition and biological activities of propolis (Ibrahim *et al.*, 2016a). The chemical composition of propolis is linked to its origin region (Popova *et al.*, 2015).

The term bee includes all propolis-producing bees, namely European honeybees (*Apis mellifera*) and Asian honeybees (*Apis cerana*, *Apis florea*, *Apis andreniformis*, and *Apis dorsata*). It also includes stingless bees, such as those of the genera *Geniotrigona*, *Heterotrigona*, *Melipona*, *Tetragonula*, and *Trigona* (Zulhendri *et al.*, 2021). Honeybee and stingless bees produce propolis, which is a complex mixture of resinous material collected by bees from

different plants and modified by their salivary secretions (Woisky & Salatino, 1998). Consequently, the characteristics of propolis are entirely dependent on the type of local flora (Bankova *et al.*, 1992). A study from Brazil showed that the stingless bee propolis from the species *Scaptotrigona bipunctata*, *Melipona quadrifasciata quadrifasciata* and *Plebeia remota* were to a certain extent, antibacterial. The bacteria include *Escherichia coli*, *Klebsiella pneumoniae*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (Surek *et al.*, 2021). Malaysian bee species which are dominant pollinators for Peninsula Malaysia are *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT) which are the common stingless bee species (locally known as kelulut) (Ibrahim *et al.*, 2016a). Many studies have shown that the greater activity of the HI propolis sample on bacteria than GT samples, can be due to its various chemical compositions and compound concentrations (Ibrahim *et al.*, 2016a). Propolis was also reported to have higher antioxidants than honey (Shehata *et al.*, 2020).

Previous studies also showed that HI propolis have large amounts of flavonoids compared to GT propolis (Ibrahim *et al.*, 2016a). Phenolic compounds and flavonoids are essential components of propolis and found to have antimicrobial effects to oral bacteria (Pasupuleti *et al.*, 2017). Recently, several studies have analysed antimicrobial activity of propolis produced by *Heterotrigona itama* (HI) of which is commonly found stingless bees in Malaysia.

In Malaysia, many studies have begun to emerge recently from 2016 to 2020. The geographical locations where the source plants might vary with respect to the local flora at the site collection and seasons may affects chemical composition of propolis, but it was concluded that bee species also play role in determining the chemical content and biological activity (Ibrahim *et al.*, 2016; Nazir *et al.*, 2018). This evidence is supported by the findings by Asem *et al.*, (2019) where propolis obtained from various species of the same bee farm (Perak) varies in antioxidant potency (Asem *et al.*, 2020). Statements regarding this issue appeared to be conflicting because some Malaysian studies concluded that bee species played a role (Ibrahim *et al.*, 2016). However, other Malaysian studies showed that geographical factors played a role (Annisava *et al.*, 2019). These studies may indicate that there are two factors which will affect the propolis chemical composition and biological activity which are propolis geographical locations (site and seasons) and the bee species. The predominating proportion of these interplay of factors remains to be investigated.

With the current existing background, our study was conducted to analyse and compare the effectiveness of antimicrobial properties of Malaysian propolis (MP). Malaysian propolis (MP) is produced by same species of stingless bee (*Heterotrigona itama*) MP from two different regions, namely Lenggong Perak and Raub Pahang were tested against *E. faecalis*. Antimicrobial Susceptibility Test (AST), Minimum Inhibitory Concentration Test (MIC) and Minimum Bactericidal Concentration Tests (MBC) were conducted.

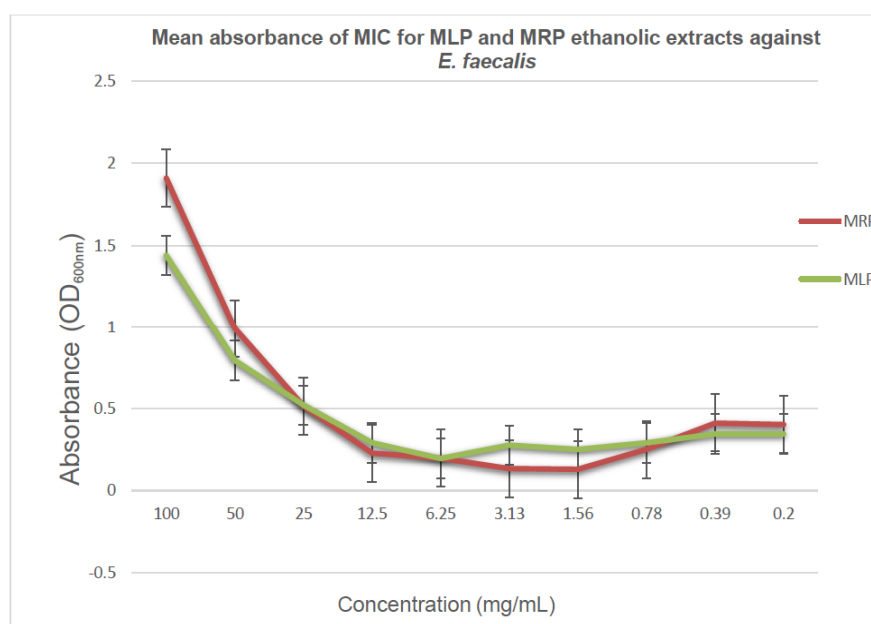


Figure 1: Mean absorbance of minimum inhibitory concentration (MIC) of MRP and MLP extracts against *E. faecalis*.

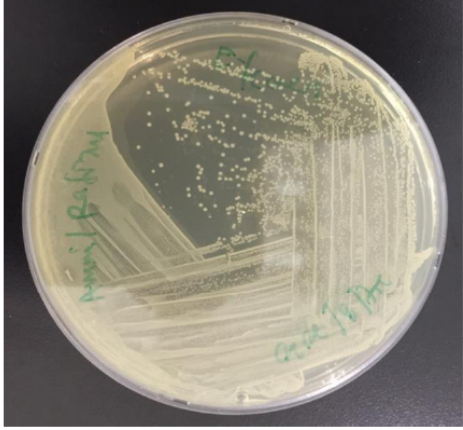
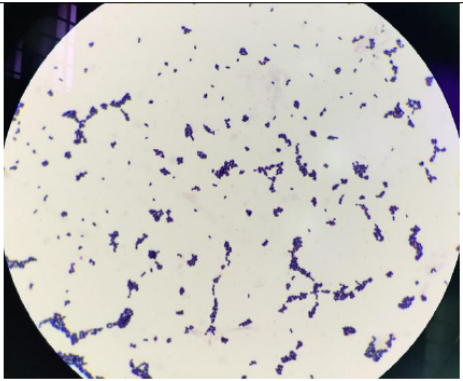
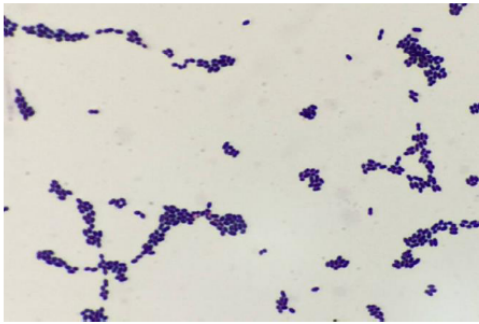
	<p>Colony morphology:</p> <p>On solid media, <i>Enterococcus faecalis</i> appear as smooth, whitish to yellowish circular convex colonies with entire edges.</p>
 	<p>Gram stain: Gram-positive cocci</p> <p>Microscopic appearance: Under 100x magnification displays diplococci and single cocci.</p>

Figure 2: Colony Morphology and Gram Staining of *E. faecalis*.

METHODOLOGY

Sample Collection and Extraction of Propolis

Raw Malaysian propolis produced by *Heterotrigona itama* (HI) were purchased from two regions namely, Lenggong, Perak and Raub, Pahang (Please refer to Table 1). The extraction followed the steps (with minor modifications) described by a study (Al-Masoodi *et al.*, 2022). Two batches of 10g were macerated and labelled (MLP-Lenggong and MRP-Raub) were soaked in 100mL of 70% ethanol and shaken (200 rpm) for 7 days, sonicated for 30 minutes under 27 °C at moderate setting. They were filtered using No.1 Whatman filter paper and left overnight at room

temperature and were then concentrated using rotary evaporator for solvent removal and stored overnight under minus 80°C. Then, it was lyophilized for 3 days (Figure 3). The lyophilized samples were stored in - 20°C freezer for further use. Figure 3 from (A) to (J) shows the procedure of ethanolic extraction of MP.

Dissolution of Propolis Extract

Propolis was dissolved in dimethyl sulfoxide (DMSO) with a concentration of less than 1%. The procedure was modified from Al Ani *et al* by increasing the concentration to 5% (Al-Masoodi *et al.*, 2022). The amount of propolis used was then dissolved in 1mL of 5% DMSO prior further analysis. This step was repeated for all antibacterial and SEM analyses.

Preparation of Bacteria Suspension

E. faecalis (ATCC 29212) was purchased from SIGMA Aldrich (USA) and was rehydrated in 0.5 mL of

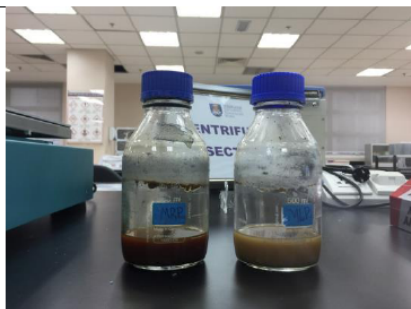
brain-heart infusion broth (BHIB) and incubated at 37°C for 18-24 hours. On the next day, glycerol stock culture was prepared by mixing 0.5mL of sterilised 50% glycerol with 0.5 mL of *E. faecalis* culture. All the



A) The raw Malaysian propolis purchased from Humaira Honey Sdn Bhd, Lenggong, Perak and Aasta Stingless Bee Honey Farm, Raub, Pahang and stored in deep freezer -80°C.



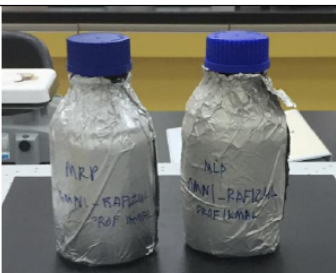
B) 10 grams of both Malaysian propolis were weighed by using weighing machine.



C) The MRP and MLP were minced and mixed with 70% ethanol in a ratio of 1:20 (propolis:ethanol).



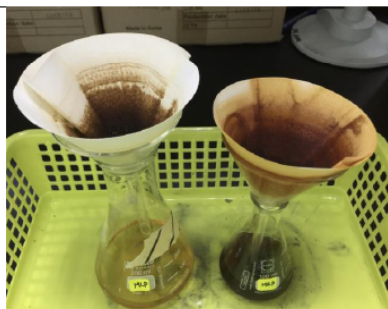
D) The MRP and MLP were mixed with ethanol using vibrator at 200rpm for 7 days.



E) The mouth of flask was covered using parafilm and the entire flask was covered with aluminium foil prior to sonication.



F) Sonication of the mixture using ultrasonic bath for 30 minutes at 27°C in moderate setting.



G) Extracted Malaysian propolis was filtered using Whatman filter paper no 1 and left overnight on the bench.



H) Evaporation of filtrate by using rotary evaporator at 40°C for 2 hours until appropriate viscosity is obtained.

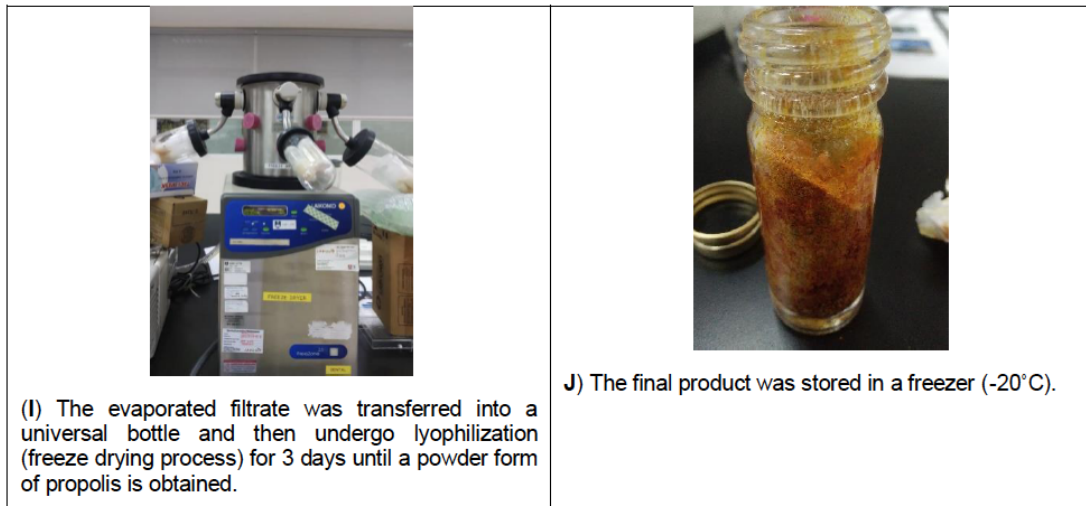


Figure 3: Ethanol extraction procedures of MP from Lenggong and Raub.

Table 1: Shows the Differences between Lenggong and Raub Farm in Terms of Stingless bee Species, Number of Hives and Types of Surrounding Plants

Farm	Stingless bee species (Bee spp studied, in bold)	Number of hives	Types of surrounding plants (Similar plants in bold)
Lenggong, Perak	1) <i>Heterotrigona itama</i> (HI) 2) <i>Geniotrigona thoracica</i> (GT)	50	1) Kelapa 2) Belimbing buluh 3) Pinang 4) Longan 5) Belimbing madu, Jambu batu, Akar Dani, Nangka, Selasih, , Pauh, Buluh china, Delima, Pagoda, Durian belanda, Forget me that, Kemangi, Tahi ayam, Limau nipis, Jeliti, Muraya, Bongor, Markisa, , Air mata pengantin, Ulam raja, Jasmin, Gelam tikus, Ru kuning, Powder puff, Misai kucing, Bottle brush, Cupiya kuning, Jasmin Thai campur, Melor, Bunga kolong, Anggur, Gading gajah merah, Pisang and Keladi.
Raub, Pahang	1) <i>Heterotrigona itama</i> (HI) 2) <i>Heterotrigona erythrogastra</i> (HE) 3) <i>Geniotrigona thoracica</i> (GT)	40	1) Kelapa 2) Belimbing buluh 3) Pinang 4) Longan 5) Dokong, Jambu air, Rambutan, Kasai/Matoa, Mangga, Kuini, Belimbing besi, Manggis, Durian, Mempelas, Senduduk, Bayam liar, pokok Bidens/jarum Sepanyol, pokok bunga air mata pengantin, Lidah kucing, ros jepun, bunga Cosmos/ulam raja, bunga Zinnia, pokok akasia, pokok balak Dipterokarpa.

*: same species or same type of plants in two different regions.

glycerol stock culture tubes were then kept at -80°C for further analysis.

Gram Staining Analysis

Gram staining for *E. faecalis* was carried out for confirming the staining for *E. faecalis* and standard protocol for Gram staining was applied (Smith & Hussey, 2019).

Calcium Hydroxide [Ca(OH)₂]

Calcium hydroxide (Calcicur ®) was purchased from

Intisar Strategies Sdn Bhd in syringe form and ready to be clinically used and tested without dilution (concentration not specified).

ANTIBACTERIAL ASSESSMENT

Antimicrobial Susceptibility Test (AST)

AST was conducted by dividing a brain heart infusion agar (BHIA) plate into four sections which are 50 and 100mg/mL of dissolved MRP ethanol extract, 5% DMSO (negative control) and Ca(OH)₂ (positive control). The plates were placed in a 37°C incubator for

18-24 hours and diameter of the inhibition zones produced by the samples was measured using a metal ruler in millimetre (mm). The test was conducted in triplicate. The same procedure was repeated substituting the MRP with MLP.

Minimum Inhibitory Concentration (MIC) Test

The MIC is defined as the concentration of samples which inhibit the visible growth of microorganisms and carried out by using a 96-well microplate dilution method. The number of bacterial suspension (*E. faecalis*) was standardized to concentration of 1×10^8 CFU/mL per well. The MRP extract was serially diluted in the 96-well plate and the final concentration of extracts ranged from 200 to 0.1 mg/mL. The microtiter plate was labelled 1 to 12 according to its column and A to H according to its row. 200 μ L (200mg/mL) of extract solution was pipetted into well number 1, 100 μ L of BHIB was pipetted into well number 2 until 12. Two-fold serial dilution was conducted starting from well number 1 to well number 10 and each well were mixed well. After that, 5 μ L of the standardized bacterial suspension was pipetted into well number 2 to well number 12. Well number 11 consists of BHIB and bacteria culture only as reference for bacterial growth and well number 12 consists of BHIB, bacteria culture and 5% DMSO as negative control. Well consisting 200 μ L of BHIB only as in row E and F starting from well number 1 until number 10 was used as reference/blank. Another reference, in row H consisting of ethanol extracts of propolis and BHIB serial dilution starting from well 1 to 10. Both constants were used as a reference when the observation for the results of the MIC were done through the naked eye. For the positive control, the same procedure was used but the dissolved propolis ethanol extract was substituted with $\text{Ca}(\text{OH})_2$. The microplates were then incubated in a 37°C incubator for 18-24 hours. The MIC values was determined by observation of the turbidity of each well. The wells that show visible growth were labelled as (G) while wells that have no growth were labelled as (NG). The lowest concentration well that shows no growth of bacteria by the naked eyes was considered as the MIC value.

Minimum Bactericidal Concentration (MBC) Test

MBC was determined by sub-culturing all the contents from the MRP and MLP extracts onto BHIA plates. Wells without visible growth (from MIC test) were selected for MBC test. All plates were incubated at 37°C for 18 to 24 hours. The concentration of the sample that showed no visible growth of *E. faecalis* was considered as the MBC.

Scanning Electron Microscopy (SEM) Analysis

The SEM was performed according to a paper by Yenugu *et al.*, 2004 with some modifications. Bacterial suspension of *E. faecalis* was prepared using the same method as mentioned above. MIC concentration for both MLP and MRP were prepared accordingly and added to the standardised bacterial suspension. The solution was then incubated for 24 to 48 hours. After incubation, the bacteria suspension was centrifuged at 10000 x g for 10 min and the supernatant was removed to obtain the pellet. The bacteria pellet was then fixed with 4% glutaraldehyde in 0.1M phosphate buffer pH 7.2 for a minimum of 1 hour or overnight. The centrifugation was repeated twice. The samples were then sent to UiTM Puncak Alam for SEM (Quanta 450 FEG, United States) analysis.

DATA ANALYSIS

Data collection was analysed by using Kruskal-Wallis for non-parametric test from Statistical Package for the Social Sciences (SPSS v26) to determine the statistically significant level. P-value of $p < 0.05$ was considered as statistically significant. The significance level was set at 5% ($\alpha = 0.05$, two-tailed). Thus, 95% confidence interval (CI) was applied. Statistical analysis was done using unpaired t-test to differentiate the difference of the two averages.

RESULTS

Gram Staining

Gram staining was conducted on *E. faecalis* ATCC 29212 strain using standard Gram staining protocol (Smith & Hussey, 2019). *E. faecalis* showed Gram-positive cocci (See Figure 2). Colony morphology of the selected bacterial was observed on BHIB agar showed circular, and convex appearance.

AST of Ethanolic Extract of MRP and MLP Against *E. faecalis*

The antibacterial susceptibility test of the ethanolic extracts of MRP and MLP were performed against *E. faecalis* by disc diffusion assay (Table 2) whereby $\text{Ca}(\text{OH})_2$ and DMSO (5%) were used as control positive and negative respectively. It has been shown that both extracts have activities against the selected bacteria. However, MLP performed bigger zone of inhibition for both concentrations used (100mg/mL and 50mg/mL) compared to MRP. The zone of inhibition for MLP was 14.67mm and 14.33mm for 100mg/mL and 50mg/mL respectively. Whereas for MRP, the zone of inhibition was 11.00mm and 10.00mm for 100mg/mL and

50mg/mL respectively. For control positive [Ca(OH)₂], the zone of inhibition was almost similar to MRP extracts which is 10.33mm. Table 2 showed the mean values of the diameter of the zone of inhibition produced by each test samples.

Table 3 shows the analysis of the AST by using Kruskal-Wallis test. From the analysis it shows a significant difference in median for the zone of

inhibition of all samples except for 5% DMSO against *E. faecalis* (p value <0.05). MLP of 100 mg/mL showed a maximum zone of inhibition with diameter median (IQR) of 16.00 (0) mm followed by 50 mg/mL of MLP with 14.00 (0) mm, 100 mg/mL of MRP with 11.00 (0) mm. While for 50 mg/mL of MRP and Ca(OH)₂, both gave the lowest zone inhibition with same diameter median (IQR) of 10.00 (0) mm, (p value <0.05).

Table 2: Zone of Inhibition of MRP and MLP Extracts Against *E. faecalis*

Sample of 20 μ L in each well of 6mm	Zone of inhibition (mm \pm)			Mean \pm SD (mm)
	Plate 1	Plate 2	Plate 3	
50 mg/mL of MRP	10.00	10.00	10.00	10.00 \pm 0.00
100 mg/mL of MRP	10.00	12.00	11.00	11.00 \pm 1.00
50 mg/ml of MLP	14.00	11.00	18.00	14.33 \pm 3.51
100 mg/mL of MLP	16.00	12.00	16.00	14.67 \pm 2.31
5% DMSO	0.00	0.00	0.00	0.00 \pm 0.00
Ca(OH) ₂	10.00	11.00	10.00	\pm 0.58

*The test was done in triplicate.

Table 3: Zone of Inhibition Shown by Each Sample Against *E. faecalis*, Analysed using Kruskal-Wallis Test

Variables	Sample	n	Median (IQR)	X ² statistic (df) ^a	P
Zone of inhibition (mm)	50 mg/mL MRP	3	10.00 (0)	14.449 (5)	0.013
	100 mg/mL MRP	3	11.00 (0)		
	50 mg/mL MLP	3	14.00 (0)		
	100 mg/mL MLP	3	16.00 (0)		
	Ca(OH) ₂	3	10.00 (0)		
	5% DMSO	3	-		

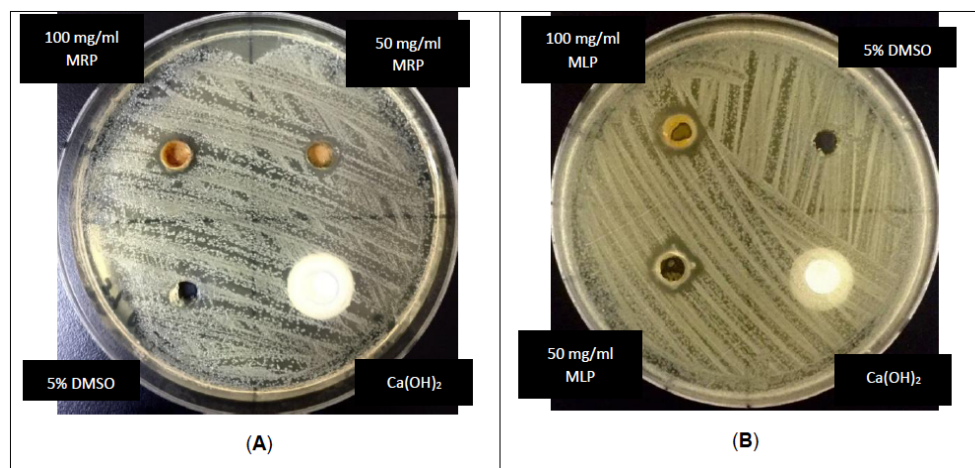


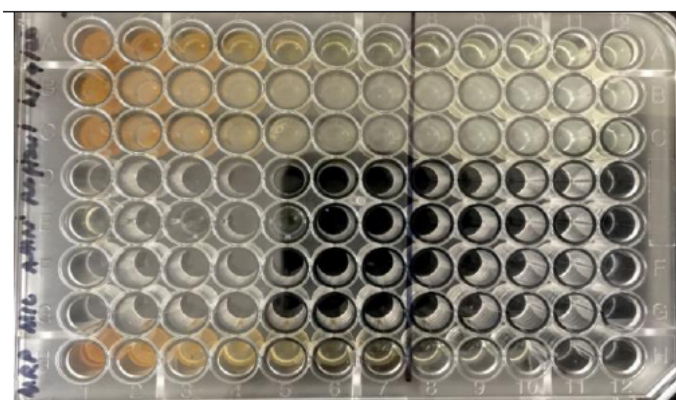
Figure 4: AST Test for MRP and MLP extracts against *E. faecalis*.

Figure A) showing antimicrobial assessment for different concentration of MRP, positive control and negative control against *E. faecalis* and Figure B) showing antimicrobial assessment for different concentration of MLP, positive control and negative control against *E. faecalis*. The diameter of zone of inhibition for all samples were shown in Table 2.

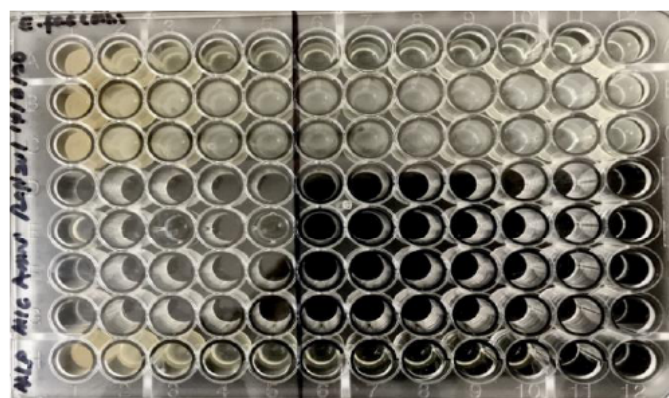
MIC of Ethanolic Extract of MRP and MLP Against *E. faecalis*

The MIC test of ethanol extracts of MRP and MLP against *E. faecalis* was conducted using 96 wells microtiter plate (shown in Table 4 and Figure 5). Based

on the observation using naked eye, the solution was clear from wells number 1 until well 6 for MRP. While for MLP, the solution was clear from well number 1 until well 5 for all triplicates tested. The MIC value was estimated based on naked eye observation for MRP and MLP was 1.56mg/mL and 6.25mg/mL respectively.



(A)



(B)



(C)

Figure 5: MIC Test for MRP and MLP extracts against *E. faecalis*.

Figure A showing MIC test for different concentration of MRP and 5% DMSO and Figure B showing MIC test for MLP and 5% DMSO. MIC test was conducted using microdilution method on a 96-well plate. Naked eye observation of the data was shown as in Table 4. While the absorbance for all mixtures were measured using microplate reader at OD 600nm. The data obtained were shown in Table 6.

Table 4: MIC of MRP and MLP Against *E. faecalis*

Sample	Concentration (mg/mL)												
	Test	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78	0.39	0.20	Positive control (Ca(OH) ₂)	Negative control (DMSO, 5%)
MRP	1	C	C	C	C	C	C	C	T	T	T	C	T
	2	C	C	C	C	C	C	C	T	T	T	C	T
	3	C	C	C	C	C	C	C	T	T	T	C	T
MLP	1	C	C	C	C	C	T	T	T	T	T	C	T
	2	C	C	C	C	C	T	T	T	T	T	C	T
	3	C	C	C	C	C	T	T	T	T	T	C	T

T = Turbid; C = Clear.

Table 5: MBC of MRP and MLP against *E. faecalis*

Sample	Concentration (mg/mL)											
	Test	100.00	50.00	25.00	12.50	6.25	3.13	1.56	0.78	0.39	0.20	Positive control (Ca(OH) ₂)
MRP	1	NG	NG	NG	G	G	G	G	G	G	G	NG
	2	NG	NG	NG	G	G	G	G	G	G	G	NG
	3	NG	NG	NG	G	G	G	G	G	G	G	NG
MLP	1	NG	G	G	G	G	G	G	G	G	G	NG
	2	NG	G	G	G	G	G	G	G	G	G	NG
	2	NG	G	G	G	G	G	G	G	G	G	NG

* The test was done in triplicate.

MBC of Ethanolic Extract of MRP and MLP Against *E. faecalis*

Table 5 shows the MBC for both propolis extracts (MRP and MLP). MIC samples from well number 1 until 7 (MRP) and well number 1 until 5 (MLP) showing clear solution were tested for MBC in-order to confirm the bactericidal concentration of both extracts. Based on the observation, MRP extract completely inhibited the growth of the bacteria at the concentration of 25mg/mL whereas for MRP, complete inhibition was observed at 100mg/mL.

ANOVA Test

Table 6 shows a comparison of the optical density (OD) reading from MIC test for both extracts (MRP and MLP) and controls. Statistical analysis using repeated measure ANOVA was conducted in-order to compare the significance difference between both extracts for all concentration. Based on the statistical analysis, the

overlapped data for both extracts at similar concentration shows no significant difference whereas with no overlapped data shows significance difference between the concentrations.

SEM Analysis

The cell morphology of *E. faecalis* before and after treatments with MRP and MLP extracts, and controls were evaluated by SEM analysis. Based on the SEM images in Figure 7, it showed that negative control (DMSO 5%) exhibited normal morphology of *E. faecalis* bacteria which showing an elongated oval shape, with measurement of an average of 1.3 μm per cell. In contrast, the cell treated with MRP and MLP extracts showed changes in the cell size, cell morphology, and cell shapes. These changes include having a more rounded structure compared to an oval, the cell wall of many of the bacteria were ruptured and seen as irregularities in their surface. Some of the bacteria exhibited complete rupture, losing their structure

Table 6: Data Analysis of MIC Test for MRP and MLP against *E. faecalis*

Sample	Concentration (mg/mL)	Absorbance (mean)	SE	95% CI	
				Lower bound	Upper bound
MRP	100.00	1.909	0.086	1.670	2.148
	50.00	0.992	0.124	0.648	1.335
	25.00	0.515	0.067	0.330	0.699
	12.50	0.228	0.015	0.187	0.270
	6.25	0.196	0.018	0.146	0.247
	3.13	0.134	0.024	0.066	0.201
	1.56	0.129	0.020	0.074	0.184
	0.78	0.250	0.045	0.126	0.375
	0.39	0.412	0.021	0.354	0.469
	0.20	0.402	0.025	0.333	0.470
MLP	100.00	1.439	0.086	1.200	1.678
	50.00	0.795	0.124	0.451	1.138
	25.00	0.521	0.067	0.336	0.706
	12.50	0.290	0.015	0.249	0.332
	6.25	0.195	0.018	0.145	0.246
	3.13	0.276	0.024	0.208	0.344
	1.56	0.251	0.020	0.196	0.306
	0.78	0.291	0.045	0.167	0.416
	0.39	0.345	0.021	0.288	0.402
	0.20	0.346	0.025	0.278	0.415

Table 6 shows the comparison of both samples (MRP and MLP) against *E. faecalis* at different concentrations using repeated-measures ANOVA.

completely, as shown in the figure. Effect of MRP on the cell morphology was more evident compared to MLP. Based on the SEM analysis, this can be seen with generalized irregularity on the surface cell wall of *E. faecalis* when treated with MRP, compared to MLP which shows little to no irregularity on the surface of the cell wall (Figure 7).

It showed that propolis extracts led to aggregation and shrinking of the bacterial cell. While some cells lose their budding stages and some cells appeared ruptured due to the activity of the extracts tested (Figure 7). From SEM analysis, it was found that Ca(OH)₂ also disrupted the bacterial cell morphology in terms of cell size as some of the cell shrinks. This is because the average length of one cell is less than 1 µm. While some cells were clumped together after being treated with Ca(OH)₂. The view of most of the bacteria were obscured by the presence of an amorphous mass of sedimentation, assumed to be caused by Ca(OH)₂. This is in comparison to 5%DMSO, MLP and MRP exhibiting a clearer view.

DISCUSSION

Bioactive compounds such as phenolic, tannin and flavonoid has been reported to contribute to antimicrobial activity against various type of bacteria. Although there are many stingless bee species that can produce propolis, HI was chosen instead of GT since HI propolis have large amounts of flavonoids compared to GT propolis, according to Ibrahim *et al.*, 2016. Their study showed that HI was the most effective in inhibiting against all tested bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Listeria monocytogen*, *Acinetobacter baumannii*, *Salmonella typhi* and *Escherichia coli*) compared to GT propolis extract due to its high phenolic and flavonoids contents (Ibrahim *et al.*, 2016a). However, one Malaysian study showed that propolis from GT has better antioxidant activity than HI (Asem N *et al.*, 2019). Although there are studies conducted previously on the antimicrobial activity of HI against *E. faecalis* there were no comparison done on the activity of HI between different bee farms. Therefore, the current study was conducted in order to compare the antimicrobial activity of propolis

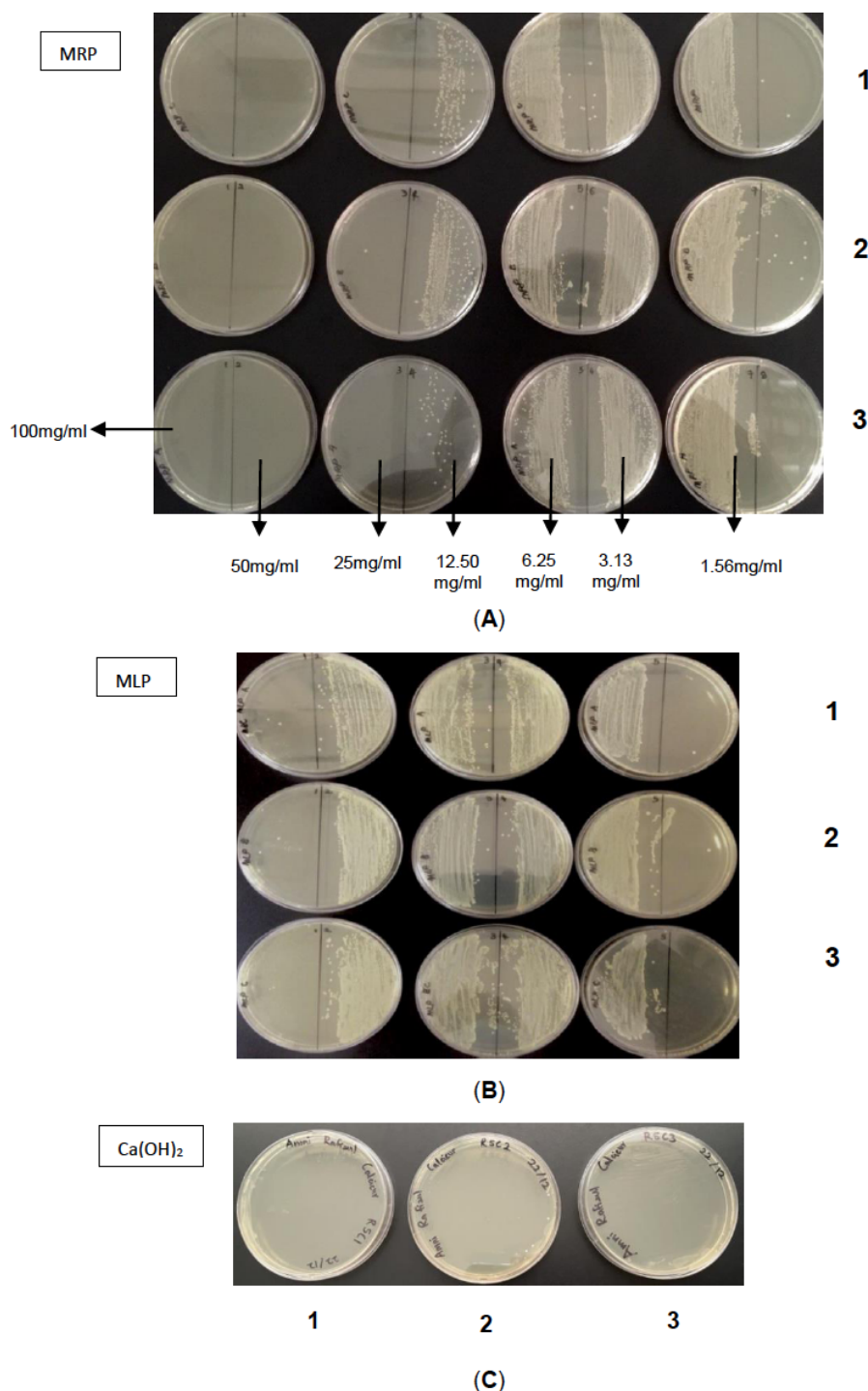




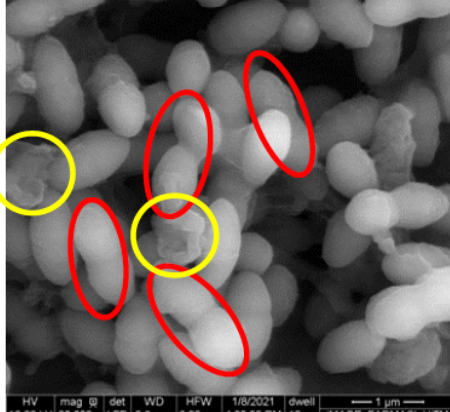
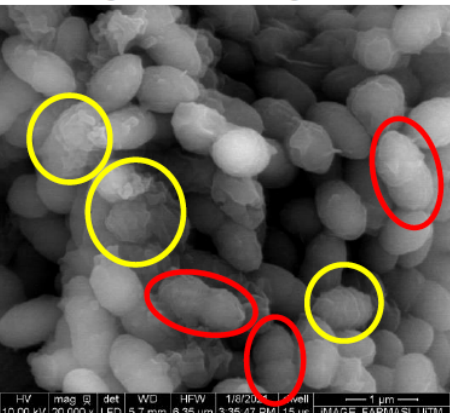
Figure 6: MBC Test for MRP and MLP extracts against *E. faecalis*.

The images show MBC test performed on BHI agar for both extracts (MRP and MLP). Clear solution from the selected wells of MIC test were tested for bactericidal activity. Figure A shows MBC test for MRP, Figure B for MLP and Figure C for Ca(OH)_2 against *E. faecalis*.

produced by HI from Lenggong and Raub against *E. faecalis*.

Antibacterial study was conducted using disc diffusion test in order to screen the effect of the extract on the selected bacteria. Based on AST analysis, we

found that MLP showed better activity against *E. faecalis* compared to MRP. However, for MIC, MBC and SEM analysis, higher antibacterial activity was performed by MRP compared to MLP. Although MLP has a bigger inhibition zone but in MIC and MBC, it requires higher concentration to inhibit the bacteria

<p>5% DMSO</p>	 <p>Image A: 20 000x magnification</p>  <p>Image B: 20 000x magnification</p>	<p><i>E. faecalis</i> generally appears as an elongated oval shape, with measurement of an average of 1.3µm per cell and some cells showed evidence of budding stage.</p> <p>Cell wall structure is intact in both photos (A and B), no rupture can be observed.</p> <p>Cells appeared to be in individual sequence without any clumping and aggregation.</p> <p>Red circle: Showing the cells in the budding stage.</p>
<p>MLP Concentration: 6.25mg/ml</p>	 <p>Image A: 20 000x magnification</p>  <p>Image B: 20 000x magnification</p>	<p><i>E. faecalis</i> generally appeared to be more rounded oval shape and measured 1.0µm.</p> <p>Image A</p> <p>Yellow circle: Some cells appeared ruptured or burst due to the effects of MLP extracts.</p> <p>Red circle: Budding stage can still be seen.</p> <p>Image B</p> <p>Yellow circle: The bacterial cell wall integrity has been compromised.</p> <p>Some cells aggregated due to the effects of MLP extracts.</p> <p>Red circle: Budding stage still can be observed but less than those observed in 5% DMSO.</p>

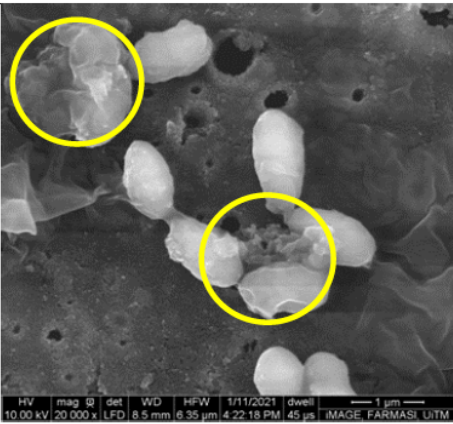
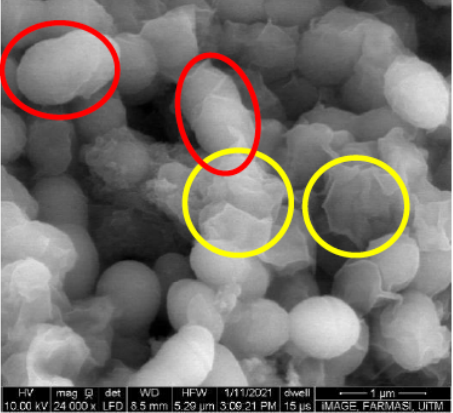
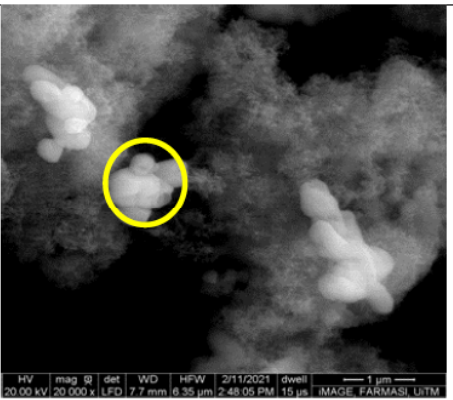
<div>MRP</div> <div>Concentration:</div> <div>1.56mg/ml</div>	<div></div> <div>Image A: 20 000x magnification</div> <div></div> <div>Image B: 20 000x magnification</div>	<div>Image A</div> <div>Yellow circle: Severe rupture is observed due to the effects of MRP extracts.</div> <div>No budding stage present.</div> <div>Image B</div> <div>Red circle: Budding stage not clearly seen or very low in number.</div> <div>Yellow circle: Some cells appeared to be ruptured and shrunk, measure 0.8-0.9 µm.</div> <div>Aggregation can only be seen in image B.</div>
<div>Ca(OH)₂</div>	<div></div> <div>Image A: 20 000x magnification</div>	<div>Ca(OH)₂ has disrupted the bacterial cell morphology.</div> <div>Yellow circle: Ca(OH)₂ ruptured the cell and severely reduced the cell size (0.5 µm).</div> <div>Ca(OH)₂ also aggregated the bacteria which caused the bacteria to be clumped together.</div> <div>There was Ca(OH)₂ sedimentation and most of the bacteria were obscured from view, showing masses of amorphous clumps.</div>

Figure 7: Scanning Electron Micrographs for MRP, MLP and Controls against *E. faecalis*.

growth. Meanwhile, for MRP it shows a smaller inhibition zone but lower concentration was required to completely inhibit the tested bacteria in MBC. This could be due to the activity of the active compounds that present in the extracts. Besides that, AST only can be used to screen the effect of the extracts at the tested concentrations. On the other hand, the minimum

inhibitory concentration and minimum bactericidal concentration test were conducted in-order to determine the lowest concentration that inhibits and kills the bacteria respectively. Next, based on the SEM micrograph, it shows that MRP shows stronger effect on the cell size and cell morphology compared MLP with minor damage of the cells.

Based on our study, we found that there is a difference in the activity of both propolis (MRP and MLP) from two different regions against *E. faecalis*. This coincides with our research hypothesis which states that there is a difference in antimicrobial properties of Malaysian propolis from HI sourced from two different regions of Peninsula Malaysia against *E. faecalis*. One of the reasons in affecting the activity for both extracts (MLP and MRP) could be due to the type of plants present surrounding the hives (Table 1). However, due to the scope of our study, we would not be able to investigate in depth on the active compounds of each plant present in the propolis collected from both regions.

Previous study Shehata *et al.*, 2020 stated that geographical region and the plants surrounding bees hives majorly affects the functional properties and the bioactive compounds present in the propolis. They also studied on the active compounds of propolis collected from six geographical regions produced variations in their bioactivity and physicochemical properties. This statement is also supported by a study by Kustiawan *et al.*, 2017, which states that the major bioactive compounds were found to be different among the 7 groups of propolis which are poplar, Brazilian green, birch, red, Mediterranean, clusia, and Pacific propolis. In Malaysia, study by Mohd-Yazid *et al.*, 2018 also indicate that the composition and quality of propolis are significantly influenced by the location where the bees were bred. *H. itama* propolis from Besut possess the best quality due to its high scavenging activity against free radical and cytotoxic activity against cancer cell compared to *H. itama* propolis from Dungun, Tanah Merah, and Gua Musang (Mohd-Yazid *et al.*, 2018). According to Ferreira *et al.*, 2017 chemical profiles of different green propolis from the same region are similar. The sharing of a plant resin source by phylogenetically distant bees (Apinae and Meliponinae) proves that bee biological variants play little role in the selection of plants for resin collection and that the availability of potential botanical sources is essential (Ferreira *et al.*, 2017).

Other than that, Mahani *et al.*, 2013 implied that different propolis from different regions (Java and Sulawesi) of same species (*Trigona* sp), showed different antidiabetic properties. This may be due to dissimilar "contributing" trees found in the farms in Sulawesi and Java. Furthermore, other studies reported that propolis collected from two different regions, two different species and two different propolis harvest time contained the same chemical compound

by Teerasripreecha *et al.*, 2012; and Kustiawan *et al.*, 2017. This is because the farm mainly grows the same type of tree. Thus, based on the previous study we suggest that type of trees does influence the chemical composition of the propolis, not by the species of sting or stingless bees (Teerasripreecha *et al.*, 2012).

Propolis samples tested in our study showed more profound antimicrobial activity against *E. faecalis* compared to Ca(OH)_2 . This was shown by the activity of both MRP and MLP showing positive antimicrobial effects against *E. faecalis*. A study by Saha *et al.*, 2015 reported that the antimicrobial properties of the propolis samples used showed the highest antimicrobial activity against *E. faecalis* compared to metronidazole, chlorhexidine in combination with *Curcuma Longa*, and Ca(OH)_2 . This study also stated that calcium hydroxide possesses the lowest antimicrobial property against *E. faecalis* (Saha *et al.*, 2015). Mirzoeva *et al.*, 1997 reported that the mechanism of antibacterial activity for propolis was mainly influenced membrane permeability and membrane potential of *E. faecalis* thereby reducing the resistance of these cells. However, unlike Ca(OH)_2 , propolis was not affected by the dentine buffering effect (Portenier *et al.*, 2003). Additionally, a randomised control trial showed that propolis (source not disclosed) is superior to calcium hydroxide in terms of their removal potency from the root canal after thorough irrigation with sodium hypochlorite (Ahmed, 2021). Referring to Table 1, the two farms exhibit some similar, as well as dissimilar plant trees, which may contribute to the differing antimicrobial properties. Future study may be required in order to explore the bioactive compounds of the plants surrounding the beehives

CONCLUSION

This study showed that MRP has a higher antimicrobial activity when compared to MLP, Ca(OH)_2 and 5% DMSO, against *E. faecalis*. Conversely, MLP showed a significant inhibition zone in AST but requires higher concentration for MIC and MBC tests.

SEM showed MRP has more profound effects on *E. faecalis* by affecting its cell morphology and cell size compared to MLP. Furthermore, SEM for Ca(OH)_2 it is recommended that other methods of comparing effects of Ca(OH)_2 on *E. faecalis* be used. Admittedly, we can dilute Ca(OH)_2 to provide clear SEM view but clinical practice protocol will not be adhered.

We can however conclude that, although the propolis are from the same species, their antimicrobial

properties from Raub differed from Lenggong, probably due to the presence of dissimilar trees and plants in these regions. We may also pave the way to “engineer” or “design” the propolis with specific beneficial properties by co-locating the hives within the vicinity of the appropriate plant source. Furthermore, the interaction between two contributing factors whether geography predominates over bee species, can be investigated in future studies. Thus, the information gathered from this study can be used for the development of new product with low cost but more effective.

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CONFLICT OF INTEREST

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

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