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Original Article

In-vitro Comparison of Antibacterial Activities on Stingless Bee Propolis using Selected Extraction Methods

Osamah Hasan Hamood Al-Masoodi[1], Hasnah Begum Said Gulam Khan[1], Izyan Hazwani Baharuddin[1] and Ikmal Hisham Ismail[1].

[1]Faculty of Dentistry, Universiti Teknologi MARA Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia.

Corresponding Author:
ikmal_hisham@uitm.edu.my
Handphone No.: +60123305002

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ABSTRACT

This study aimed to compare the different selected extraction methods of propolis extracts against Enterococcus faecalis (E. faecalis). Extraction of Malaysian Propolis (MP) from Heterotrigona itama, was carried out using 70% ethanol. For the selected extraction processes, such as, centrifugation-assisted extraction (CAE), vacuum-assisted extraction (VAE), and shaking-assisted extraction (SAE) methods were used. Antimicrobial activity against E. faecalis was assessed using the antibacterial susceptibility test (AST). The results showed that SAE, followed by CAE, had better antimicrobial properties as compared to those obtained by VAE. The inhibition zones for SAE, CAE, and VAE were 5.3mm, 4.67mm, and 4.16mm, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for extracted MP by SAE were 12.5 and 25 mg/mL, respectively. Furthermore, non-setting calcium hydroxide's [$Ca(OH)_2$] MIC and MBC were determined to be 50 and 100 mg/mL, respectively. As a result, we believe that extraction of raw MP from Heterotrigona itama using the SAE method was more effective than $Ca(OH)_2$ against E. faecalis.

Keywords: *Malaysian propolis, Enterococcus faecalis, $Ca(OH)_2$, Antimicrobial activity, Heterotrigona itama*

INTRODUCTION

Bees are social insects, well recognized for their pollination and honey production abilities that live in hives. Bees are classified into two broad groups: those that sting (honeybees) and those that do not sting (stingless bees). While stinging bees are more well-known, taxonomic research has determined that stingless bees are the world's oldest bees (Al-Hatamleh et al., 2020). Both bees produce propolis, which has been hailed as a natural alternative to pharmaceuticals since it is expected to have fewer

adverse effects. (Garg et al., 2014). Sting bees such as *Apis mellifera, Apis cerana, and Apis dorsata* produce honey, bees' wax, and propolis, whereas stingless bees such as *Heterotrigona itama and Geneotrigona thoracica* produce just honey, bee bread, and propolis. Certain bee species contribute to the chemical and biological characteristics of specific propolis (Annisava et al., 2019; Ibrahim, Mohd Niza, et al., 2016; Ibrahim, Zakaria, et al., 2016).

Propolis or bee glue, is formed both by honeybees and stingless bee species incorporating from a variety of plant sources including leaves, flowers, sap, resin and buds (Ibrahim, Zakaria, et al., 2016).

Most stingless bees are found in tropical and subtropical regions, and over 500 species have been identified. *Heterotrigona itama, Geniotrigona thoracica, Tetragonula laeviceps, Lepidotrigona terminata,* and *Tetragona apicalis* are the most commonly reported stingless bees in Malaysia (Kelly et al., 2014). Malaysian stingless bees create two forms of propolis: which are sticky and hard (Awang et al., 2018).

H. itama and *G. thoracica* are the most common propolis producers in Malaysia. They generate sticky propolis with a high concentration of flavonoids, which has been shown to have antibacterial properties (Awang et al., 2018).

Heterotrigona itama and *Geniotrigona thoracica* are the two most widely domesticated stingless bee species in Malaysia. In both urban and rural regions, *H. itama* is commonly found (Hamid et al., 2016). In addition, according to a previous Study, 90% of the honey from stingless bees sold in Malaysia comes from *H. itama* (Kelly et al., 2014). The *Meliponini tribe* includes the stingless *H. itama* bee, which belongs to the *Trigona genus* and is part of the *Meliponini subtribe.* The Malaysian community refers to it as “lebah kelulut” (Yusop et al., 2018).

Additionally, propolis includes a variety of different secondary plant metabolites, the quantities of which vary according to the season, geographic origin of the collection, and proximity of a beehive to certain plant sources (Alvarez-Suarez, 2017; Ibrahim, Zakaria, et al., 2016). Numerous phenolic compounds, including flavonoids, aromatic acids, and their esters, are often collected by honeybees from poplar buds (*Populus sp*.). These elements are found in high concentrations in poplar buds and have been shown to have a variety of biological and pharmacological characteristics. Numerous research groups have demonstrated that poplar propolis provides antibacterial against both gram-positive and gram-negative bacteria, including multidrug-resistant organisms (K. Wang et al., 2014).

Propolis contains natural compositions of diverse secondary metabolites that have a variety of bioactive properties, including antibacterial, anti-angiogenic, antiulcer, anti-inflammatory, antioxidant, and antiviral properties. In its raw state, it is made up of 45–55 percent plant resin, 25–35 percent wax, 5–10 percent essential and aromatic oils, 5% pollen, and 5% other natural materials (de Figueiredo et al., 2015). Polyphenols (flavonoids, phenolic acids, and esters) and phenolic aldehydes and ketones are common in propolis (Elnakady et al., 2017; Król et al., 2013) . Resins and vegetable balsam account for half of the total, followed by bee wax (30%), pollen (5%), essential and aromatic oils (10%), and many other ingredients, including organic compounds (Abdulkhani et al., 2017). Propolis has a wide range of materials depending on the period and location of its gathering (Afrouzan et al., 2017).

When utilized in different ways, propolis has a significant positive impact on human health. Antibacterial, antifungal, anti-inflammatory, antiviral, anesthetic, and antioxidant (Omar et al., 2017), antitumoral, antiprotozoal, anticancer (Sforcin, 2016), antihypertensive, anticarcinogenic, and anti-hepatotoxic in addition to cytotoxic activity are just a few of the many uses it has today (Toreti et al., 2013).

Honeybees, in contrast to stingless bees, use wax to build their nests or combs, whereas stingless bees use plant resins or propolis. It's for this reason that stingless bees have been shown to generate more propolis than any other bee species (Mohd Suib et al., 2021; Popova et al., 2021).

There are two ways to look at the antibacterial properties of propolis. First, it has to do with the microbe itself, and second, it has to do with the immune system's stimulation, which in turn activates the body's natural defenses (Sforcin & Bankova, 2011). To understand how it affects microorganisms, researchers have looked at the activity of propolis. This has allowed them to deduce that it has an influence on permeability, membrane potential disruption, and ATP synthesis, as well as lowering bacterial motility. Propolis' antibacterial properties seem to be more effective against Gram-positive bacteria than Gram-negative bacteria. Gram-negative bacteria's outer membranes are species-specific, as they produce hydrolytic enzymes, which may break down the active components in propolis (Sforcin, 2016).

The persistence of oral microorganisms including *Actinomyces spp*., *Enterococcus spp*., *Propionibacterium spp*., and fungi like *Candida sp*. following initial endodontic therapy has been linked to failed endodontic treatment (Pinheiro et al., 2003). A study suggests that, coronal leakage of root filling following primary endodontic treatment, may have allowed bacteria to penetrate and re-establish themselves (Endo et al., 2014). According to bacterial cultures and scientific analyses, root canal treatment failures are frequently infected with *E. faecalis* (Barbosa-Ribeiro et al., 2016; Endo et al., 2014; Pinheiro et al., 2003). Therefore, *E. faecalis* was selected for this study. According to the World Health Organization (W.H.O.), a variety of remedy may be obtained from medicinal plants. Since various studies have demonstrated propolis's strong antibacterial efficacy against *E. faecalis* in unsuccessful root canal therapy, it has emerged as a promising beneficial alternative. One previous study showed that *E. faecalis* growth and proliferation were hindered by propolis (Oncag et al., 2006). Due to its ability to resist *E. faecalis*, it has been widely suggested as an intracanal medication (Amir et al., n.d.; Awawdeh et al., 2009; Victorino et al., 2009). In addition, propolis was found to be more efficient than calcium hydroxide against *E. faecalis* after both application for 1 and 2 days (Awawdeh et al., 2009; Victorino et al., 2009), whereas Madhubala et al. found that propolis had a 100% efficacy after 2 days of treatment (Madhubala et al., 2011).

It is not possible to utilize raw propolis directly for analysis or treatment at all. In order to dissolve and release the most potent components, they must first be extracted. Extractant agents include ethanol, methanol, water, hexane, acetone, dichloromethane, and chloroform (Miguel et al., 2010). So as to make use of propolis' bioactive components, it is necessary to extract them to produce high-quality and cost-effective propolis-containing products, thus, it is crucial to select a suitable extraction technique in order to produce high-quality and cost-effective propolis-containing products. The main composition of propolis varies greatly because honeybees gather propolis from a variety of plants in various geographic and climatic regions. Propolis has a variety of chemical profiles, and the ideal extraction method for one species of propolis may not work for another (Bankova et al., 2019).

Propolis, on the other hand, has low water solubility and is soluble in organic solvents because resins are largely apolar, regardless of their chemical composition. Because plants have an insoluble cellular matrix, the extraction of propolis differs from that of medicinal plants. Extracting propolis' bioactive ingredients from bee glue seeks to dissolve key plant-derived chemicals and remove the wax, which is always found in propolis (up to 20%) (Bankova et al., 2021).

It has been shown that ethanol is the best solvent for extracting bioactive components from propolis, such as flavonoids and polyphenols. The ethanol/water (70:30, v/v) combination solvents are recommended because they are considered green solvents (Chattopadhyay, 2015; Escriche & Juan-Borrás, 2018). A 70% ethanol extract of Malaysian propolis has the potential to be a good antioxidant agent (Usman et al., 2016). Not only does solvent type affect extraction efficiency, but so do temperature, duration, and the size of the propolis particles in the extract (Sawaya et al.,

2011). Another study stated that maceration is the most common method for making propolis extracts, but ultrasound-assisted extraction produced good results and dramatically accelerated the process (Trusheva et al., 2007).

The chemical composition of propolis vary greatly because bees gather propolis from a variety of plants in various geographic and climatic regions. Propolis has a variety of chemical characteristics, and the ideal extraction method for one species of propolis may not work for another (Bankova et al., 2019). Propolis has a low water solubility but is soluble in organic solvents because resins are largely apolar, regardless of their chemical composition. Due to the presence of insoluble cellular matrices in plants, extracting propolis is different from extracting therapeutic herbs. Extracting propolis' bioactive ingredients from bee glue seeks to dissolve key plant-derived chemicals and remove the wax, which is always found in propolis (up to 20 % of wax) (Kubiliene et al., 2015). One study by Pobiega et al, compared various means of extraction methods and studied the extracted propolis's antimicrobial activities. The methods investigated were: shaking extraction (SE), ultrasound-assisted extraction (UAE), and ultrasound-assisted shaking extraction (SUAE). They showed that SUAE had better antimicrobial activity compared to SE or UAE alone.

There were various methods of extraction as outlined by studies above and to our knowledge there is lacking in a standard propolis ethanolic extraction method that we can apply to any future studies investigating stingless bees. Some methods appears to be suitable for antimicrobial testing, while others are suitable for other analyses for example, on anticancer. Furthermore, studies are also lacking regarding extracting propolis from different stingless bee species.

Pobiega et al carried out investigations on antimicrobial effects displayed by stinging bee propolis from Poland. So, this method by Pobiega et al was adapted with modification and the comparison was carried out between previous techniques used for extraction stingless bee propolis, in Faculty of Dentistry, Universiti Teknologi MARA. From previous literature, we must carefully select the extraction method in order to obtain high-quality and more effective propolis-based active components, particularly looking at antimicrobial properties.

Thus, the aim of our study was to compare the antimicrobial susceptibility test (AST), the minimum inhibitory concentration (MIC), and the minimum bactericidal concentration (MBC) of ethanolic extract of Malaysian propolis using different extraction methods against *E. faecalis*.

MATERIALS AND METHODS

Collection of Propolis Sample:

The raw Malaysian Propolis produced by the stingless *H. itama* bee was obtained in Raub, Pahang and stored in a deep freezer (-80 °C).

Preparations of dry ethanolic extracts of propolis (EEP):

Ten grams of minced raw propolis was first weighed and diluted 1:10 (w/v) in 100 mL of 70% ethanolic solution. Propolis was subsequently extracted by using three distinct extraction techniques (Pobiega et al., 2019a). In the first adopted method, which is shaking assisted extraction (SAE), the sample was shaken (200 rpm) at 28 °C for 7 days (SK-600 shaker, Jeiotech, Korea). Then, samples were sonicated for 30 min at 27°C at a moderate setting (Ultrasonic, Jeiotech, Korea). All samples were filtered using a Whatman No. 1 filter and subsequently condensed at 40 °C under reduced pressure (Rotavapor R-210, Buchi, Switzerland). In the second adopted method, which were centrifugation assisted extraction (CAE) after evaporation one sample was subjected to centrifugation. The third extraction method was added and modified from Pobiega

study and propolis was subjected to the vacuum for 30 minutes (VAE). Then, all samples from the three methods were lyophilized for three days to create a powdered form of propolis.

The final product for all samples were stored in a freezer (-25 °C). Reconstitution of the dry propolis powder was carried out by dissolving in dimethyl sulfoxide (DMSO) before further analysing the resultant varied concentrations. Figure 1 shows the process of extraction methods and Table 1 tabulates the three different methods.

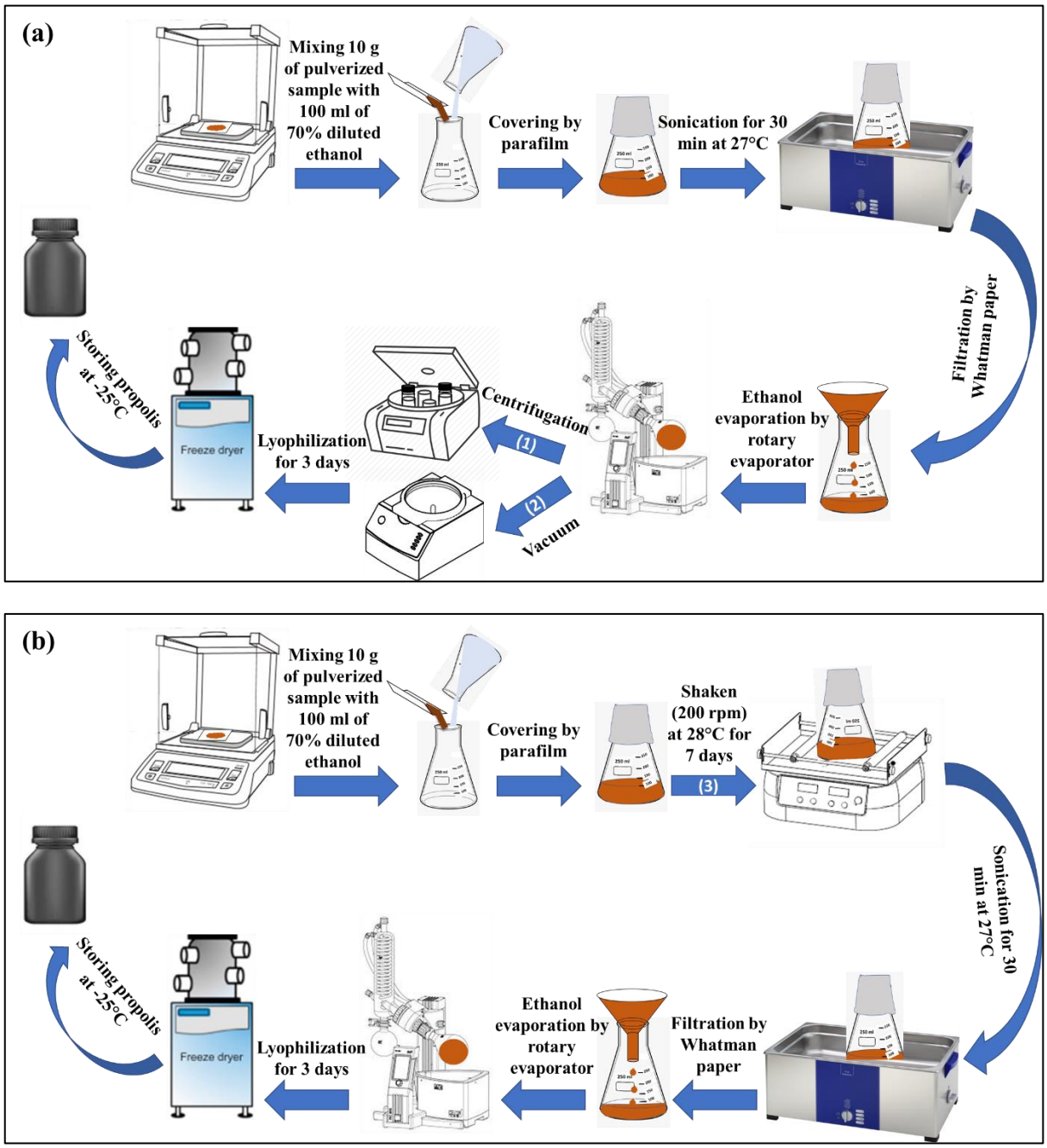

Figure 1: Schematic diagram of the preparation of dry ethanolic extracts of propolis using (a) centrifugation assisted extraction (CAE) and vacuum assisted extraction (VAE), and (b) shaking assisted extraction (SAE) methods.

Table 1: Different extraction techniques used for the preparation of propolis extracts.

Sample / Process	10 mg/ mL with **CAE**	10 mg/ mL with **VAE**	10 mg/mL with **SAE**
Shaken for 7 days	-	-	√
Sonication	√	√	√
Filtration	√	√	√
Ethanol evaporation	√	√	√
Centrifugation	√	-	-
Vacuum	-	√	-
Lyophilization	√	√	√

Note: The CAE, VAE and SAE refer to centrifugal-assisted extraction, vacuum-assisted extraction, shaking- assisted extraction respectively.

Preparation of Non-setting Calcium Hydroxide [$Ca(OH)_2$]

Calcium hydroxide powder was purchased from Dentalkart Sdn Bhd. To achieve a paste-like consistency, $Ca(OH)_2$ powder was mixed with sterile saline in a 1.5:1 (wt/vol) ratio.

Preparation of Bacterial Suspension:

Preparation of Stock Culture

E. faecalis (ATCC 29212) was purchased from Next Gene Scientific Sdn Bhd and rehydrated in a vial containing 0.5 mL of brain heart infusion broth (BHIB). The stock culture was prepared by pipetting 0.5 mL of brain-heart infusion broth and 0.5 mL of 50% glycerol into cryovial tubes. Then, followed by pipetting 0.1 mL of inoculated BHIB into each of the cryovial tubes, respectively. Some of the cryovial tubes were stored as stock culture. Only one 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 were noted as showing positive results on the growth of bacteria. Another five stocks of a culture of *E. faecalis* were prepared from the grown bacteria on the blood agar. All the cryovial tubes consisting of 25% glycerol stock (final concentration) were stored in a deep freezer (-80˚C).

Confirmation of the bacterial culture and preparation of bacterial suspension

Frozen glycerol stock culture was thawed, and BHI broth was used to make a fresh bacterial suspension and the inoculated broth was incubated for 18-24 hours at 37˚C. The next day, Gram staining was performed to examine the bacterial cell morphology. Colony morphology of *E. faecalis* was observed by growing the bacteria on brain heart infusion (BHI)

and blood agar (BA). Bacterial culture was also streaked on the brain heart infusion (BHI) broth and blood agar (BA) and then incubated for 18-24 hours at 37°C. Observation and recording of the colony morphology was done on the next day. Both tests were conducted to confirm the purity of the bacterial stock used for the study. The bacteria number was standardized to 0.08 to 0.13 using a spectrophotometer (OD_{600nm}) for every antibacterial assay, which is equivalent to 1 x 10^8 CFU/mL (Humphries et al., 2018).

Antibacterial Assay

Antimicrobial Susceptibility Test (AST)

Antibacterial susceptibility test of propolis extracts against *E. faecalis* was conducted using agar diffusion method. Bacterial suspension with a standard number of bacterial cells (0.08 to 0.13 using a spectrophotometer (OD600nm), as mentioned in the previous section was used for this assay.

BHI agar BHI agar was prepared according to instructions provided by the manufacturer. Bacterial suspension with a standard number of bacterial cells as mentioned in the previous section was used for this assay. 100 µL of bacterial suspension was pipetted on the BHI agar plate and spread evenly using a sterilized cotton swab. Then the plates were allowed to dry for 30 minutes before the creation of wells using a cork-borer (6 mm diameter) on the agar medium. The bottom of the plates was labeled accordingly and 50 mg/mL of the ethanol extract of Malaysian propolis (EEP) was placed in the first well followed by 50 mg/mL of calcium hydroxide [Ca(OH)2] in the second well and 5% of Dimethyl sulfoxide (DMSO) in the third wells. $Ca(OH)_2$ and 5% DMSO were used as control positive and negative respectively. All experiments were conducted in triplicates. After 18-24 hours of incubation, all plates were observed for the zone of inhibition. The zone of inhibition surrounding the agar wells was measured in mm unit using Vernier caliper and the average values were calculated.

Minimum Inhibitory Concentration (MIC) Test

Minimum inhibitory concentration (MIC) is the lowest concentration of the test sample to inhibit the visible growth of microorganisms incubated at 37°C between 18-24 hours, which was how it was measured. A 96-well microtiter plate was used for MIC assay (Munir et al., 2020; S. Wang et al., 2017). The plate was labeled accordingly. Fifty mg/mL of EEP propolis was prepared using 5% DMSO (final concentration) as a stock starting concentration. The amount 200 µL of the extract was pipetted into the first well, then 100 µL of BHIB was pipetted into the second well until well number 12. The amount 100 µL of the extract from the first well, transferred into the second well, for the two-fold dilution. The sample was mixed thoroughly, and this step was repeated until well number 10. The remaining 100µL was discarded. Then, 100 µL of the standardized *E. faecalis* bacterial suspension was pipetted into wells 1- 10, resulting in a total volume of a solution of 200µL in each well. Well number 11 consisted of 100 µL broth and 100 µL organism combination, which was used to observe bacterial growth. While well number 12 consisted of only broth, for sterility control. The same technique was repeated for [$Ca(OH)_2$] at a concentration of 50 mg/ml and 5% DMSO. The microtiter plate was incubated at 37°C for 18-24 hours. The lowest concentration well that showed no visible growth of bacteria via naked eyes, was considered as the MIC value. The experiment was repeated three times to determine the mean value of the MIC.

Minimum Bactericidal Concentration (MBC) Test

MBC test was conducted by sub-culturing the test sample from the MIC plate on a fresh BHI agar plate. All plates were then incubated for 18-24 hours at 37°C. On the next day, the lowest concentration of the extract with no bacterial growth was considered as MBC. All triplicates' data were recorded and tabulated.

Statistical Analyses

Statistical 6.0 from StatSoft (Tulsa, OK, USA) was used to analyze the results statistically and to show significant differences between the means. The mean (n=9) and standard error of the mean is used to express the results. The level of significance for all statistical procedures was set at $p<0.05$. The data are reported as the mean standard deviation (SD) in triplicate.

RESULTS

Antibacterial Susceptibility Test

Antibacterial susceptibility test using different extraction methods of the ethanolic extract of Malaysian propolis was conducted against *E. faecalis*. Table 2 shows the zone of inhibitions (ZOIs) of 50 mg/ml of propolis with the different extraction methods, 50 mg/ml of calcium hydroxide, 100 % of DMSO, and 50 % of DMSO. Based on the results of the Kruskal-Wallis test. The inhibitory zone mean sizes of the samples were significantly different after 24 hours ($P<0.05$). The mean size inhibitory zone of propolis (50 mg/ml) with extraction method using SAE is higher than other extraction methods using VAE and CAE, which are 4.67mm and 4.16mm respectively. While for positive control, 50 mg/mL of $Ca(OH)_2$ showed lower zone (4.16mm) compared to 50mg/mL propolis using shaken method (SAE). Whereas, for negative control (5% DMSO), no inhibitory zone was observed surrounding the wells (Figure 2).

Table 2: Antibacterial susceptibility test of 50 mg/mL of propolis with different extraction methods, 50 mg/mL calcium hydroxide, 100 % of DMSO, and 5 % of DMSO.

Sample	Zone of inhibition (ZOI) (mm)			
	1	2	3	Mean ± SD
Propolis 50 mg/ml with CAE	4.0	4.5	4.0	4.16 ± 0.29
Propolis 50 mg/ml with VAE	4.5	5.0	4.5	4.67 ± 0.29
Propolis 50 mg/ml with SAE	4.5	5.0	6.5	5.30 ± 1.04
Calcium hydroxide 50 mg/ml	4.0	4.0	4.5	4.16 ± 0.29
DMSO 100%	3.0	3.0	4.0	3.33 ± 0.58
DMSO 5%	0.0	0.0	0.0	0.0 ± 0.0

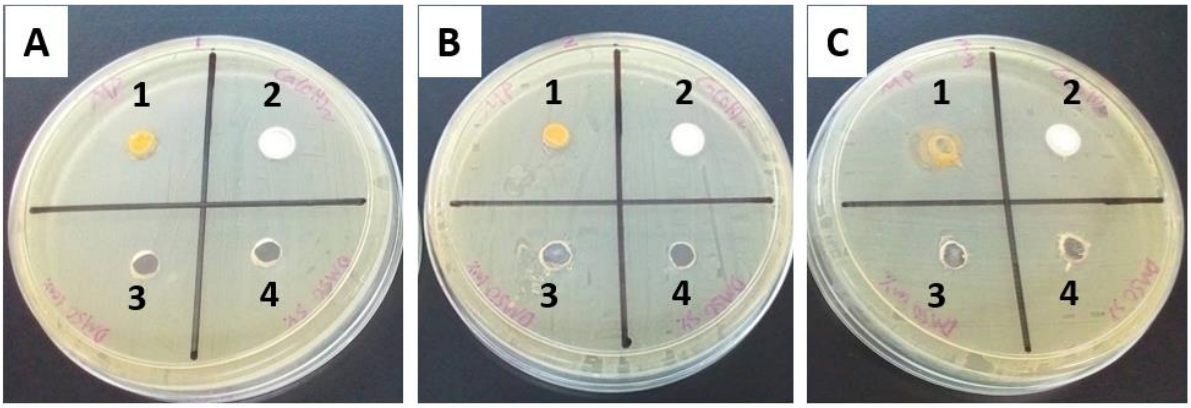

Figure 2: ZIO of (1) 50 mg/ml of propolis, (2) 50 mg/ml calcium hydroxide, (3) 100 % of DMSO and (4) 50 % of DMSO. The propolis extraction method using (A) CAE, (B) VAE, and (C) SAE.

Determination of DMSO Concentration as Solvent

A 96-well microtiter plate was used for determining the safe concentration of DMSO as a solvent using the broth microdilution method. The plate was labeled accordingly. Then, 100 µL of BHI broth was pipetted into the second well until well number 12. A 200 µL of 100% of DMSO was pipetted into well number 1. Two-fold serial dilutions were performed by pipetting 100 µL of the DMSO from well number 1 and transfer to well number 2. The sample was mixed thoroughly, and this step was repeated until well number 10. The remaining 100 µL was discarded. Then, 100 µL of the standardized bacterial culture of *E. faecalis* was pipetted into well numbers 1 to 11, resulting in a 200 µL volume of solution in each well. Well number 11 has a positive control with a mixture of 50 µl BHIB and 50 µl organism. Well number 12 has a negative control with only BHI broth. Observation of the MIC value was conducted using a spectrophotometer at 600nm wavelength. The MIC test was conducted to obtain a suitable concentration of DMSO to be used as a solvent for propolis extract. The optical density was recorded and tabulated as shown in Table 3. The observation using the naked eye was not conducted due to the changes in the cloudiness of turbidity being too close to each other. The percentage of bacterial growth was calculated by the following formula:

$$\text{The growth percentage} = \frac{\text{Mean of } \mathrm{OD}_{600} \text{ microplate result}}{\text{Mean of} + \text{ve control culture well}} \times 100$$

Figure 3 shows the percentage of the optical density against DMSO concentration. The optical densities indicate the number of bacteria in the solution. Based on Table 3 and Figure 3, DMSO concentration which showed no inhibitory effect against *E. faecalis* is starting from 6.25% and below. The MBC test was also conducted for DMSO using the samples from the MIC test. It showed that DMSO 6.25% and below did not kill the bacteria tested (Figure 4).

Table 3: Optical density of DMSO against *E. faecalis*

DMSO [%]	100	50	25	12.5	6.25	3.14	1.56	0.78	0.39	0.2	+ con	- con
Mean	0.07	0.08	0.23	0.47	0.547	0.53	0.57	0.55	0.55	0.55	0.57	0.06
SD	0.01	0.01	0.12	0.02	0.004	0.079	0.05	0.03	0.03	0.03	0.001	0.01

Note: The MIC test was conducted to obtain a suitable concentration of DMSO to be used as a solvent for propolis extract. + control is $Ca(OH)_2$ and -ve control is 5% DMSO.

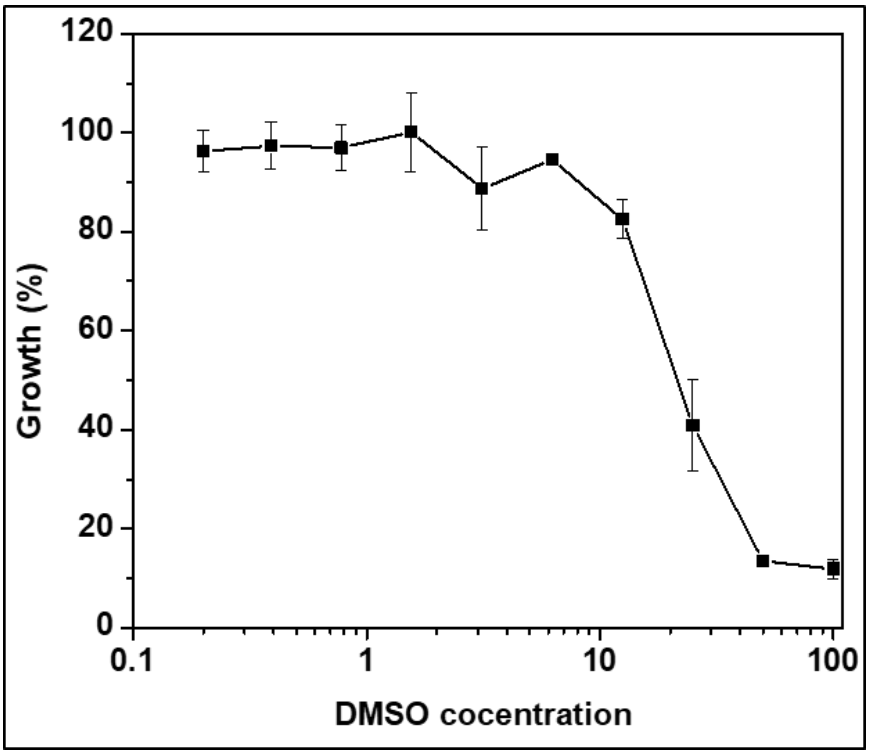

Figure 3: Growth percentage of *E. faecalis* in different concentrations of DMSO.

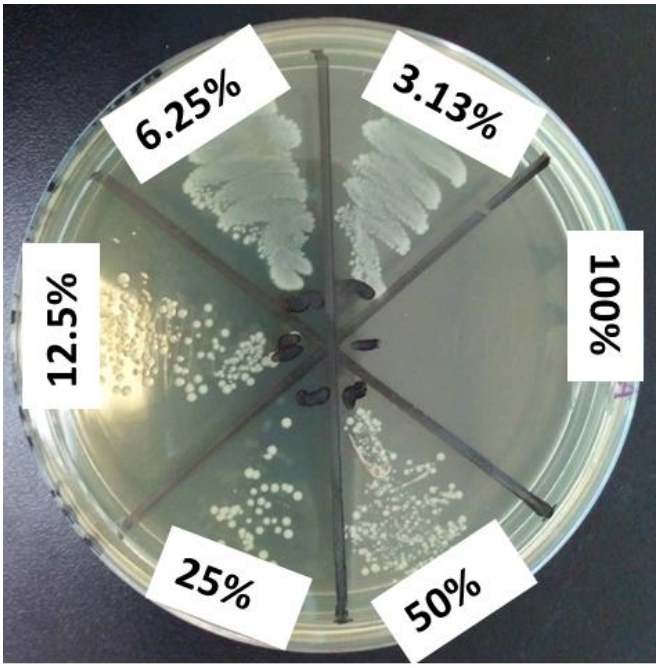

Figure 4: MBC of different concentrations of DMSO against *E. faecalis*

MIC of Propolis against *E. faecalis*:

Figure 5 illustrates the schematic diagram of the determination of MIC of the different concentrations of propolis. The MIC of the extracted propolis with SAE has the lowest concentration which is about 12.5 mg/mL compared to the other extraction methods of propolis, CAE, and VAE, in which the MIC is 25 mg/mL. Figure 6 shows the mean of OD_{600} of *E. faecalis* growth in different concentrations of propolis using a different method.

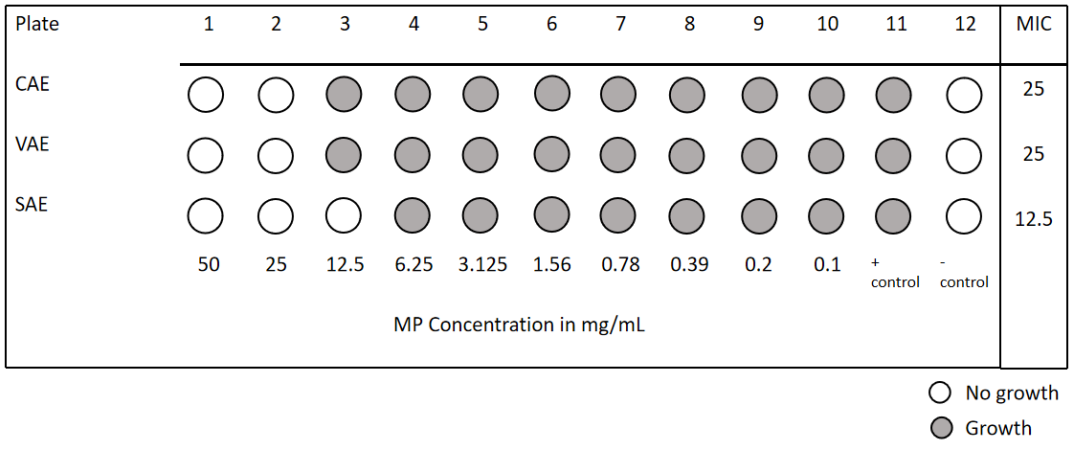

Figure 5: Schematic diagram of 96 wells microplate for MIC measurement of propolis using different extraction processes of propolis.

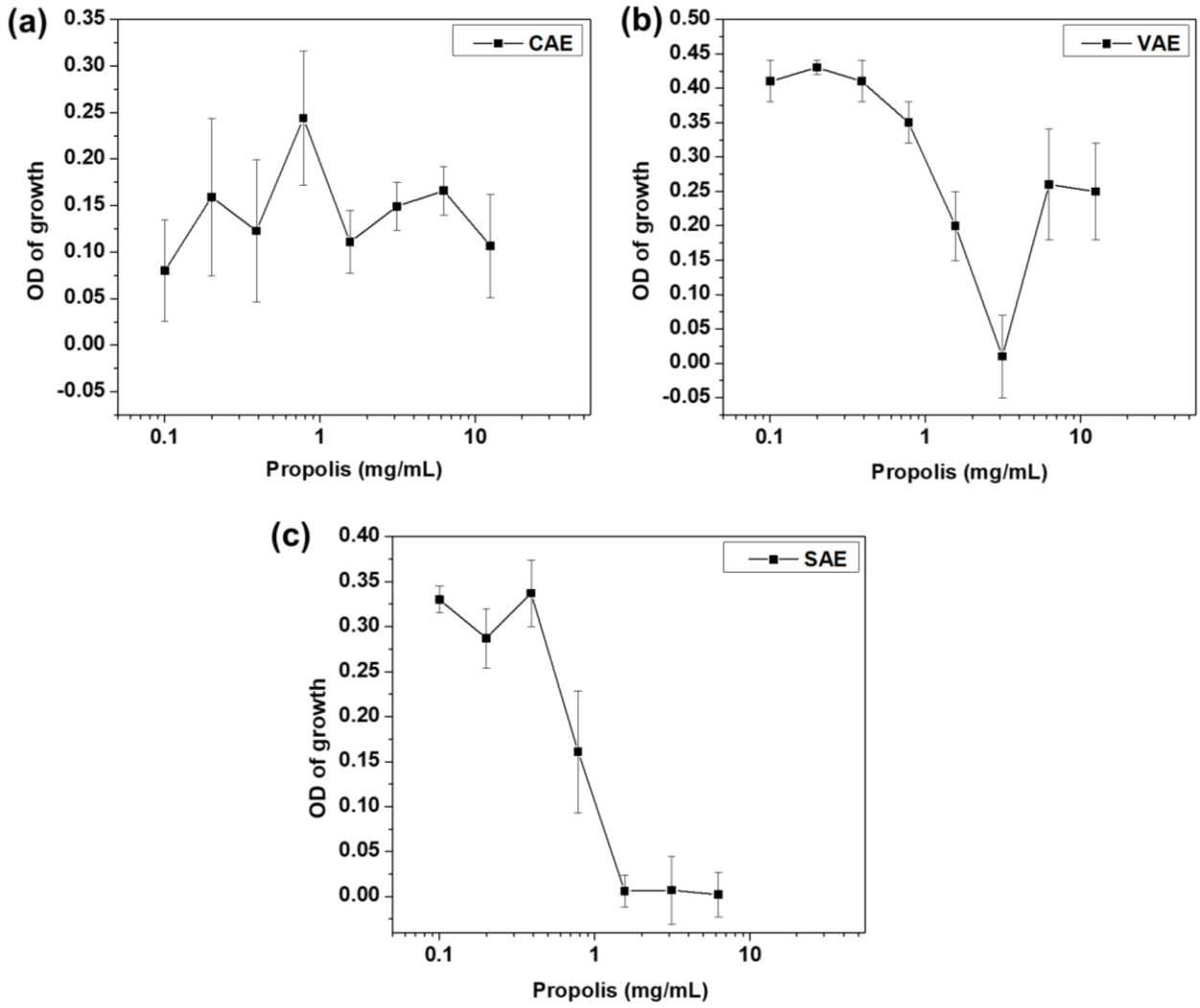

Figure 6: Mean OD_{600} of *E.faecalis* growth in different concentrations of propolis using a different method. (a) CAE, (b) VAE, and (c) SAE.

MBC of propolis against *E. faecalis*

Samples from the MIC test were used for the MBC test. Samples from well number 1 until 8 were streaked on fresh BHI agar medium. The growth of the bacteria was examined after 18-24 hours and as shown in Figure 7. The MBC of extracted propolis with SAE has the lowest bactericidal concentration, which is 25 mg/mL compared to the other extraction methods, CAE and VAE, which is 50 mg/mL.

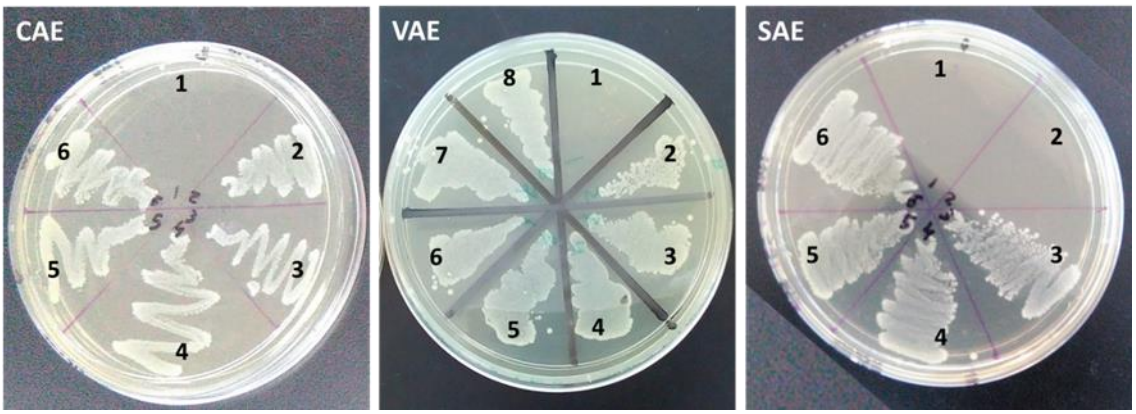

Figure 7: MBC of propolis against *E. faecalis* using different extraction methods. Where the numbers 1- 8 indicated the concentration of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39mg/mL, respectively.

MIC of $Ca(OH)_2$ against *E. faecalis*

Figure 8 shows the schematic diagram of the determination of MIC of the different concentrations of $Ca(OH)_2$. The MIC of $Ca(OH)_2$was 50 mg/mL. Figure 9 shows the optical densities of *E. faecalis* growth in different concentrations of $Ca(OH)_2$.

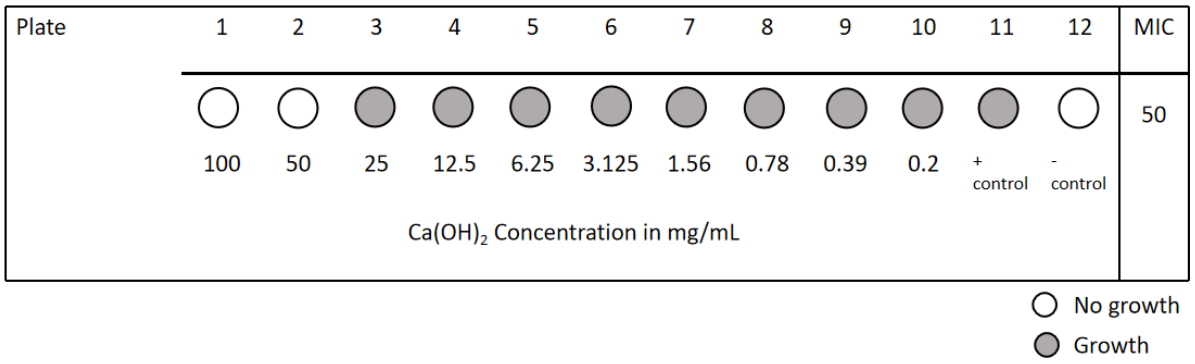

Figure 8: Schematic diagram of 96 wells microplate for MIC measurement of different concentrations of $Ca(OH)_2$.

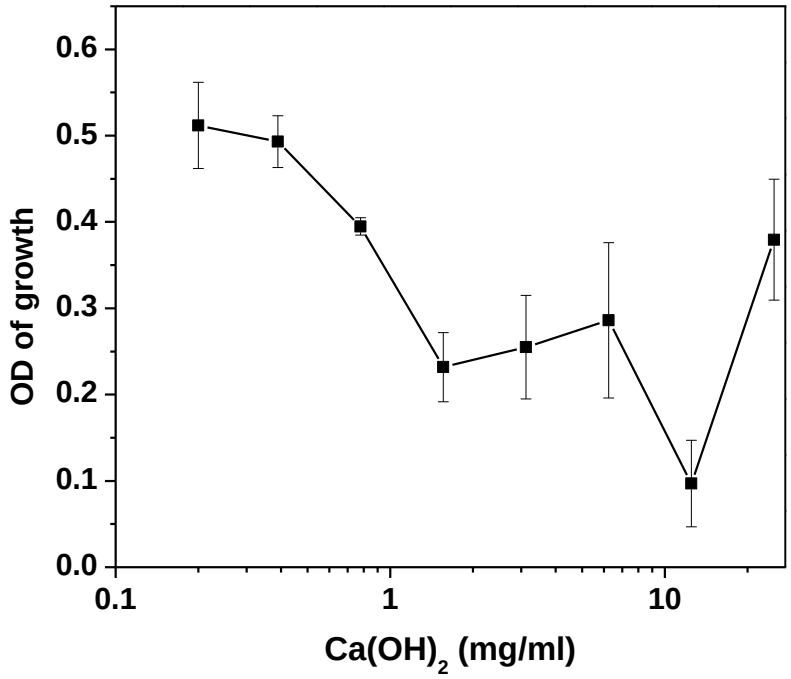

Figure 9: Mean OD_{600} of *E. faecalis* growth in different concentrations of $Ca(OH)_2$.

MBC of $Ca(OH)_2$ against *E. faecalis*

Samples from the MIC test were used for the MBC test. Samples from well number 1 until 8 were streaked on fresh BHI agar medium. The growth of the bacteria was examined after 18-24 hours and as shown in Figure 10. The MBC of $Ca(OH)_2$ is 50 mg/mL.

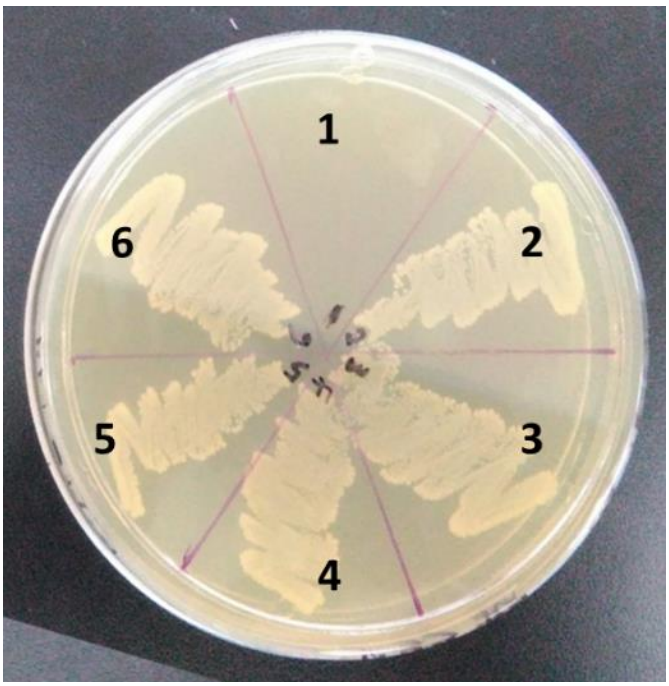

Figure 10: MBC of $Ca(OH)_2$ against *E. faecalis*

DISCUSSIONS

If propolis extracts are going to be used in food, they need to have a high level of biological activity. Choosing the right way to make crude propolis extracts is so critical. Propolis extracts produced by CAE, VAE, and SAE were compared for antibacterial activity in our research. **The antibacterial activity of SAE was shown to be greater than that of other extraction procedures, CAE and VAE.** This was due to the higher inhibitory zone of SAE as compared to CAE and VAE. According to the previous studies, shaking extraction method followed by 20 min of sonication increased the extraction of phenols by 15 and 23% and the extraction of flavonoids by 8 and 14%. The content of phenols and flavonoids determines the antimicrobial and antioxidative activity of propolis (Jug et al., 2014; Pobiega et al., 2019b). The current study compared the antibacterial characteristics of various extraction methods of Malaysian EEP against *E. faecalis*, which may be regarded a limit, as it is better to measure effectiveness against bacterial biofilm. *E. faecalis* is the primary pathogen responsible for endodontic failure (Nasim & Hemmanur, 2021).

It was observed from a study, that the propolis extracted from the sonication technique, has more potent antibacterial and antioxidant activity and so this technique was used in all three different extraction methods in our study, to achieve a potent propolis extract against *E. faecalis* (Khacha-ananda et al., 2013). According to previous research, ultrasound-aided extraction was better than 2-day shaking extraction in terms of flavonoids extraction, but less effective in terms of phenolic compounds extraction than shaking. Although the content of bioactive compounds such as flavonoids and phenols in the propolis extracts differed, no significant effect of these differences on the zone of inhibition of extracts were observed, as all extracts inhibited bacterial growth against *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Proteus mirabilis* with similar activity levels (Moncayo Luján et al., 2018). These may be due to the difference in propolis source and synergistic effect of another active component of propolis as different geographic property affects the chemical composition and biological activity of propolis (Kujumgiev et al., 1993).

Similar to Yeo et al., a higher bioactive compound content extract obtained in ultrasound-aided extraction yielded a growth inhibition effect that was only slightly higher for *Staphylococcus epidermidis*, *B. subtilis*, and *E. coli* than an extract obtained in the traditional, 1-day maceration process (Yeo et al., 2015). The complex composition of propolis extracts and interactions between the effects of components that may be present even at extremely low levels may explain this occurrence (Bankova et al., 2019). **Although most researchers assume that the propolis to ethanol ratio has no effect on extracting most propolis components, it is difficult to explain how the ethanol percentage of the extraction mixture affects the amounts of bioactive compounds found in the final extract** (Khacha-ananda et al., 2013; Trusheva et al., 2007)**.** Moreover, using a high concentration of ethanol had no effect on the percentage of extract yield. However, the use of ethanol at 70 % was better compared to the use of ethanol at a concentration less than 50 % or water because unrequired lipid wax was extracted by 70 % ethanol (da Silva Cunha et al., 2004). According to their research, *Melipona quadrifasciata* and *Tetragonisca angustula* were both used in the study to gather ethanolic extracts of propolis (EEP) using 70% ethanol. Furthermore, geopropolis extracts were found to be more effective against Gram-positive bacteria (*Staphylococcus aureus*, MSSA and MRSA, and E. faecalis) than Gram-negative bacteria (Klebsiella pneumoniae, Escherichia coli). The Melipona species outperformed the other two geopropolis samples studied, which were *Melipona quadrifasciata* and *Tetragonisca angustula* (Torres et al., 2018).

A previous study compared the three extraction methods (Maceration extraction, Ultrasound extraction (UE), and Microwave assisted Extraction (MAE)). In addition, MAE and UE techniques were found to be more efficient than maceration extraction in terms of extraction yield, time, and labour requirements (Trusheva et al., 2007). One study showed that Ethanolic Extract of Green Propolis (EEP) extraction was more efficient than Aqueous Extract of Propolis (EAP) (Paviani et al., 2011). Our study showed that the longer the shaken process, the stronger the inhibitory effect of extracts against test microorganisms. This phenomenon may be explained by the content of flavonoids and phenols being higher for longer shaken times. Therefore, further studies using Gas chromatography/mass spectrometry

(GC/MS) analysis of MP extracts from *H. itama* are recommended to determine the main contents that are responsible for its biological activity.

CONCLUSION

Antibacterial properties are influenced by the extraction process. SAE followed by CAE yielded more antimicrobial activity than VAE. DMSO had a recommended maximum concentration of 6.25 percent to be used as a solvent. *E. faecalis* was shown to be inhibited by the MP with the SAE method more effectively than $Ca(OH)_2$ and DMSO. The results of the MIC and MBC tests revealed that the SAE method of extracting propolis was superior to the other selected extraction methods.

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