STINGLES BEE PROPOLIS EXTRACT AS AN ACTIVE INGREDIENT IN ANTIBACTERIAL OINTMENT FORMULA

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ABSTRACT

Stingless bees, also known as kelulut or klanceng (in local languages), are now widely cultivated in Indonesia. Stingless bees produce honey and propolis, which is the residue from honey extraction and is more abundant than honey itself. In this study, the processing of honey sacs or propolis into active ingredients for antibacterial ointments was optimized, specifically to address skin diseases caused by the bacteria Staphylococcus aureus and Streptococcus mutans. Propolis was extracted using aqua dest and 96% ethanol and optimized using three methods, namely sonication, maceration, and decoction, and antibacterial testing was carried out on S. aureus and S. mutans test bacteria. Antibacterial testing was carried out on the extracts using the diffusion method and the microdilution method. The results of the testing showed that propolis extract was most effectively extracted with water solvent, which had a bacterial inhibition concentration of 1,000–8,000 µg/mL. Water-based propolis extract can be used as a raw material for antibacterial ointments with a usage concentration of 5–15% and has been proven effective in inhibiting bacterial growth as observed using a Scanning Electron Microscope (SEM).

Keywords: Antibacterial, propolis, ointment, scabies disease, stingless bee

Introduction

Stingless bees have different names in each region due to the diversity of tribes and languages in Indonesia (Priawandiputra *et al.*, 2020). Indonesia has the greatest diversity of social bees in all of Asia, especially stingless bees. Currently, 46 species of stingless bees are known throughout Indonesia, although records are incomplete and additional diversity is likely to exist throughout the region. Honey from stingless bees, particularly Tetragonula laeviceps, is harvested by squeezing, which produces waste in the form of honey sacs that contain a lot of propolis.

Propolis is a natural substance collected by both stinging honey bees and stingless bees. Propolis, also known as bee glue or bee cement, is a substance produced by bees from resin collected from trees and shrubs, combined with beeswax and secretions from the bees' salivary glands, which are rich in enzymes (Sawicka et al., 2012). Propolis has a complex composition of compounds and a broad spectrum of activity. Propolis has been tested on more than 600 strains of bacteria. Propolis has greater activity against Gram-positive bacteria such as Staphylococcus aureus than Gram-negative bacteria such as Escherichia coli, and the antimicrobial activity of propolis varies in different regions of the world (Przybyłek & Karpiński, 2019).

Therefore, it is necessary to optimize the processing of honeycomb waste from stingless bees (propolis) as a raw material for antibacterial ointments. Thus, this article reported the effectivity of propolis extract as a raw material for antibacterial ointments.

Methods

Propolis Extraction

Propolis was extracted using aqua dest and 96% ethanol and optimized using three methods: sonication, maceration, and decoction.

Antibacterial Test of Propolis Extract

The antibacterial test was conducted using two methods, namely the diffusion method and the microdilution method. The diffusion method was used to analyze the antibacterial potential of propolis extract using various methods. The microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Commercial propolis was used as a reference in the antibacterial test, after first evaporating the solvent used. Commercial propolis was poured into porcelain dishes and evaporated with a water bath (Memmert) until dry. Propolis extract was dissolved in DMSO (10%) and a propolis extract stock was made at a concentration of 100,000 µg/mL. Before testing, the propolis extract was dissolved with DMSO in a microtube at a concentration of 10,000 ppm. The DMSO control was made at a concentration of 10% (v/v) in an aqueous solvent.

Formulation and Method of Propolis Ointment Production

The formulation of propolis ointment with a hydrocarbon base consists of 5%, 10%, and 15% propolis extract, 3% solid paraffin, 5%, 10%, and 15% white petrolatum, 2% white beeswax/cera alba, 5% stearyl alcohol, 0.02% methyl paraben/nipagin, and 100% water. Each jar contains a total of 10 g of ointment. Each ingredient is weighed on a Petri dish and porcelain using an analytical balance. In stage I, stearyl alcohol, white beeswax, and solid paraffin are combined in porcelain and melted using a water bath (Memmert) at a temperature of 80ºC. Stage II: White petrolatum and methyl paraben are mixed, then stirred until homogeneous, then added to the mixture in stage I and stirred until homogeneous. Stage III: The mixture from stage II is poured into porcelain containing propolis extract and cooled, then packaged into pots.

Antibacterial Test of Ointment Using the Disk Diffusion Method

Each ointment formula was dissolved in 3% Tween 80% with a propolis extract concentration of 10,000 µg/mL. Test bacteria Staphylococcus aureus and Streptococcus mutans aged 24 hours, 100 µL, were inoculated onto the surface of BHI Agar using a sterile cotton bud. Sterile disc paper (Oxoid) was placed on the surface of the BHI Agar medium. A 25 µL solution of ointment from each formula was dripped onto the disc paper. Ampicillin 10 µg discs were used as positive controls, while 3% Tween 80% was used as a negative control. The media were incubated at 37 °C for 24 hours. The inhibition zones formed were observed and measured with a caliper.

Antibacterial Testing of Ointments Using Electron Microscopy

Each ointment formula was dissolved in 3% Tween 80% with a propolis extract concentration of 10,000 µg/mL. The ointment solution was diluted again in BHI medium at a concentration of 2,000 ppm. Test bacteria S. aureus and S. mutans aged 24 hours were used as test bacteria. Sterile 12 mm diameter coverslips were placed in a 24-well plate. Test bacteria solutions of S. aureus and S. mutans at a density of 107 CFU/mL, 250 µL each, were added to the wells that had been covered with coverslips. Then, propolis ointment solutions with formulas of 5, 10, and 15% propolis extract were added at 500 µL each to obtain a final extract concentration of 1,000 ppm. Wells containing solutions of both bacteria plus BHI medium without ointment were used as negative controls. The plates were incubated at 37 °C for 24 hours. After incubation, the medium in the plate wells was removed. The coverslips in the wells were washed 3 times with 1000 µL of sterile PBS. The coverslips were then fixed with graded ethanol, namely 70% for 10 minutes, 80% for 10 minutes, 90% for 10 minutes, and absolute ethanol for 5 minutes. The coverslip was then air-dried before observation with a scanning electron microscope (SEM). Before SEM observation, the coverslip containing bacteria was coated with Au and observed with SEM (Hitachi). SEM observation was performed at BRIN Gunungkidul at a magnification of 1,000–10,000x.

Results and Discussion

Propolis Extraction

The ointment formula containing natural ingredients such as propolis begins with extracting the active compounds contained in the propolis extract. This extraction is intended to take the active ingredients from the natural ingredients themselves, removing unnecessary substances, as well as making storage and transportation of the extract more efficient because it does not require a large space. The extraction is carried out using sonication, which allows for maximum extraction because it uses ultrasonic waves so that the active ingredients from propolis are more easily released. The extraction uses polar solvents, namely water and 70% ethanol. In this study, commercial propolis extract was used as a comparison. The raw materials of propolis, water extract, ethanol extract, and commercial propolis extract are shown in Figure 1.

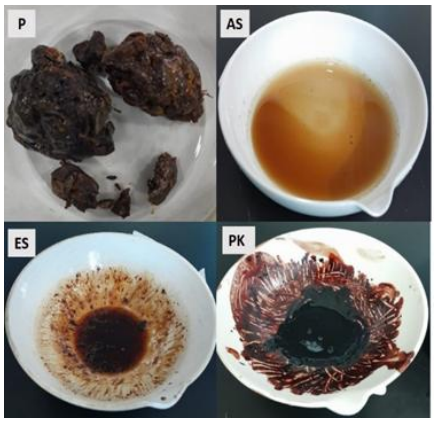

Figure 1. Propolis raw material from Tetragonula laeviceps stingless bees (P), water propolis extract using sonication method (AS), 70% ethanol propolis extract using sonication method (ES), and commercial propolis (PK)

Extract Yield

The extraction results using water solvent and 70% ethanol solvent are shown in Table 1. Based on yield weight, the propolis extraction method using 70% ethanol solvent has a higher yield compared to water solvent. In another study, lemon peel extract using ethanol solvent also had a higher yield (Melia *et al.*, 2018). Active compounds such as flavonoids, phenolics, tannins, terpenoids, saponins, alkaloids, glycosides, and reducing sugars were also more abundant in ethanol extracts (Karina et al., 2020). However, ethanol also has several disadvantages, such as a strong taste and adverse reactions. Conversely, water solvents have higher antioxidant activity than ethanol (Bruno *et al*. 2020). The next step is to test the antibacterial effectiveness to determine which extract is more active and can be used as an ointment.

Table 1. Propolis extract yield using the sonication method

Solvent	Raw material (g)	Extract (g)	Yield (%)
Akuades	50	8.09	16.18
Etanol 70%	50	11.48	22.96

Paper Disc Diffusion Antibacterial Test

The method used in testing antibacterial activity is the agar diffusion method using paper discs with the aim of determining the diameter of the inhibition zone formed around the paper disc containing the extract after an incubation period of 1x24 hours. In the antibacterial testing method, the extract sample will diffuse from the paper disc to the BHI (Brain Heart Infusion) medium that has been inoculated with test bacteria. In this study, propolis extract was dissolved in 10% DMSO solvent

at a test concentration of 250 µg/disc with ampicillin antibiotic as a positive control and 10% DMSO as a negative control.

Antibacterial testing of stingless bee propolis extract showed variations in the inhibition zone data. The inhibition zone data for propolis extract using the sonication method is presented in Figure 2 and Table 2.

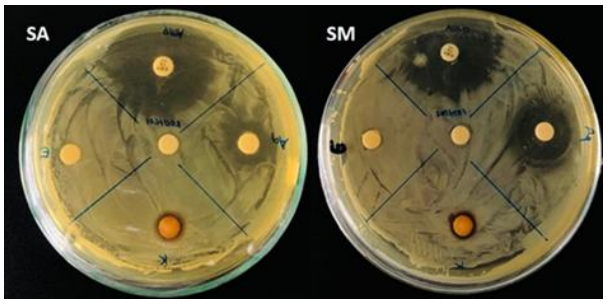

Figure 2. Antibacterial test using the paper disc diffusion method. Description: (Sonicated propolis extract in distilled water (Aq), sonicated propolis extract in ethanol (E), commercial propolis extract (K), positive control ampicillin (10 µg) (Amp), and negative control 10% DMSO (control) on Streptococcus mutans (SM) and Staphylococcus aureus bacteria (SA)

Table 2. Antibacterial test results of propolis extract against pathogenic bacteria Streptococcus mutans and Staphylococcus aureus

Test bacteria	Diameter of the brake zone for each disc paper (mm)			
	250 µg of propolis aqua extract	250 µg of propolis ethanolic extract	250 µg of commersial propolis	10 µg of ampicillin
Streptococcus mutans	17.74 ± 0.62	7.41 ± 0.06	9.78 ± 0.04	35.54 ± 3.14
Staphylococcus aureus	17.07 ± 0.19	8.30 ± 0.11	8.99 ± 0.11	34.56 ± 1.61

Based on Figure 2 and Table 2, the inhibition zone diameter of aqueous propolis extract on S. mutans bacteria was higher (17.74 mm) than that of commercial propolis extract (9.78 mm) and ethanol propolis extract (7.41 mm). The antibacterial test results show that sonicated aqueous propolis extract is more effective in inhibiting the growth of S. mutans bacteria than 70% ethanol extract and commercial extract. The sonicated aqueous propolis extract on S. aureus bacteria (17.07 mm) was higher than the commercial propolis extract (8.99 mm) and ethanol propolis extract (8.30 mm). The results showed that the aqueous propolis extract was more effective in inhibiting the growth of S. aureus bacteria. However, the antibacterial activity of propolis extract on both bacteria was still lower than the ampicillin antibiotic control at 10 µg. The size of the inhibition zone formed in the antibacterial test was likely due to differences in the characteristics of each extract. This was because of the content of compounds or active substances in propolis extract that have antibacterial properties. In this case, the active substances referred to are flavonoids, phenolics, tannins, terpenoids, saponins, alkaloids, glycosides, and reducing sugars (Karina et al., 2020).

Microdilution Antibacterial Test

The microdilution antibacterial test aims to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The extract concentrations used were 250, 500, 1,000, 2,000, 4,000, and 8,000 µg/mL. MIC was determined based on the lowest concentration that began to inhibit the growth of the test bacteria. MIC can be determined by visual observation based on the level of turbidity. MIC is the concentration at which bacteria do not grow (appears clear). In this study, the method used was adding MTT reagent. MIC was determined based on the MTT color that did not change (remained yellow, did not turn purple), which indicated that bacteria did not grow (resembling a negative control/without bacteria). Growing bacteria can change MTT from yellow to purple (resembling a positive bacterial control).

The MIC values are shown in Table 3. Based on the determination of KHM values, the aqueous extract had the lowest MIC value for both test bacteria compared to the ethanol and commercial extracts. Thus, the aqueous extract effectively inhibited both test bacteria. An extract can be said to be effective if it can inhibit bacteria at the lowest concentration. The lower the concentration of extract needed to inhibit bacteria, the more effective the extract is. The MIC value is determined by growing bacterial solutions at various test concentrations from each microplate well into a growth medium. The MIC value is determined from the concentration at which bacteria do not grow in the medium.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values

Parameter	Test Bacteria	Propolis Extract			Antibiotic Control
		Commercial	Aquadest	Ethanol	Ampicillin
MIC (µg/mL)	*Streptococcus mutans*	1.000	250	2.000	<12.5
	Staphylococcus aureus	2.000	250	4.000	<12.5
MBC (µg/mL)	*Streptococcus mutans*	8.000	1.000	>8.000	<12.5
	Staphylococcus aureus	>8.000	>8.000	>8.000	<12.5

Inhibition Curve and Time-Kill Test

Based on the growth curve in Figure 5, the aqueous propolis extract can kill both test bacteria, as indicated by the growth curve below the bacterial control. The curve shows that the aqueous propolis extract inhibits the growth of *S. aureus* starting at 4 hours at all concentration levels. Thus, the minimum inhibitory concentration (MIC) value against *S. aureus* for the aqueous propolis extract (distilled water) based on the curve is 500 ppm. However, the inhibitory effect on *S. mutans* bacteria is lower than that on *S. aureus*, meaning that *S. mutans* bacteria are more difficult to kill. In the *S. mutans* kill time curve, it can be seen that at all concentrations of water propolis extract (aqua), the growth is below that of the control bacteria, but it has not been completely killed because there is still an increase in growth. Thus, aqueous propolis extract is more effective at inhibiting and killing S. aureus bacteria compared to *S. mutans*. This is in accordance with the MIC values in Table 3, where the water propolis extract is higher in *S. mutans* bacteria than in S. aureus. In a previous study, it was concluded that ethanol propolis extract can inhibit the growth of *Streptococcus mutans* bacteria at concentrations of 40% (8 mm), 60% (9.3 mm), and 80% (11 mm) (Bruno *et al*. 2020).

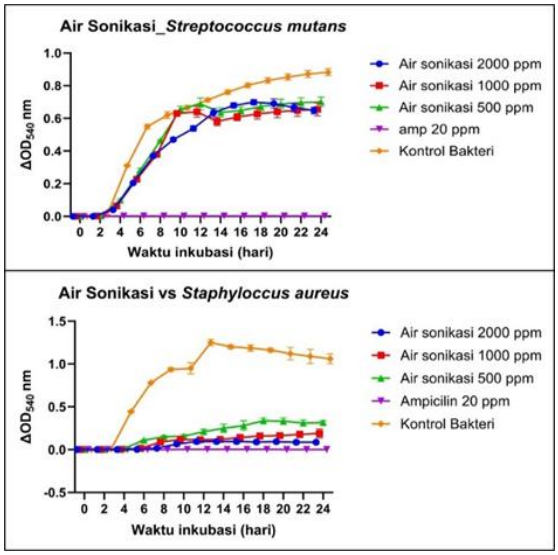

Figure 3. Testing the inhibition curve and killing time of propolis water extract (distilled water) on S. mutans and S. aureus bacteria

Ointment Formulation

The aqueous propolis extract was then formulated into an ointment preparation with the formula shown in Table 4. The propolis ointment formulation with a hydrocarbon base consisted of 5%, 10%, and 15% propolis extract ; 3% solid paraffin ; 5%, 10%, and 15% white petrolatum; 2% beeswax/cera alba, 5% stearyl alcohol, methyl paraben/nipagin 0.02% to form 1 pot contains a total of 10 g of ointment. Table 4 shows the composition and percentage of components along with the weight of each ointment formula. Based on Figure 6, ointments with higher concentrations of propolis produced a darker brown color compared to those with lower concentrations of propolis.

Table 4. Propolis water extract ointment preparation formula

Component	Formulation		
	F1 (5%)	F2 (10%)	F3 (15%)
Propolis extract 5, 10, 15%	0,5 g	1,0 g	1,5 g
Solidum paraffin wax3%	0,3 g	0,3 g	0,3 g
Vaseline	8,498 g	7,998 g	7,498 g
Beeswax white/*cera alba* 2%	0,2 g	0,2 g	0,2 g
Stearil alcohol 5%	0,5 g	0,5 g	0,5 g
Methyl paraben/Nipagin 0,02%	0,002 g	0,002 g	0,002 g
Total weight (g)	10 g	10 g	10 g

Figure 6. Propolis ointment products containing 5, 10, and 15% propolis extract in water (distilled water) using sonication method

Antibacterial Test of Propolis Ointment Using the Diffusion Method

The antibacterial activity of propolis ointment using the paper disc diffusion method is shown in Table 5. Based on Figure 7, it is known that the 10% propolis extract formula has a greater antibacterial effect on *S. aureus* bacteria (20.06 mm), while the 5% propolis extract formula is more effective on *S. mutans* bacteria (20.44 mm). Thus, higher concentrations of propolis extract do not correlate with increased antibacterial activity. Table 5 shows that 5% propolis ointment only has an effect on *S. aureus* and *S. mutans* bacteria. Meanwhile, 10% and 15% propolis ointments do not have an antibacterial effect on *S. mutans*. This is consistent with the data in Figure 5, where *S. mutans* is more difficult to inhibit and kill than *S. aureus*.

Table 5. The inhibition zone of propolis extract ointment on test bacteria at an extract concentration per ointment preparation of 250 µg/disc

Tested Bacteria	Inhibitory zone of propolis extract ointment (mm)			3% Tween 80
	Formula 5%	Formula 10%	Formula 15%	
Streptococcus mutans	9,93 ± 0,46	20,06 ± 0,38	18,76 ± 0,06	-
Staphylococcus aureus	20,44 ± 1,52	-	-	-

Observation of Ointment Inhibition on Test Bacteria with a Scanning Electron Microscope

Confirmation of the antibacterial power of propolis ointment was then carried out using SEM. The concentration of propolis extract in the ointment tested was 1,000 ppm. The inhibition of propolis ointment on both test bacteria, *S. mutans* and *S. aureus*, is shown in Figure 7. The figure shows that the bacterial control (A) contains both test bacteria in cocci form. The test bacteria without ointment application appear to grow more densely than those with ointment application (B–D). The application of 5% propolis ointment already had an inhibitory effect on growth, where the bacteria did not grow as densely. The application of 10% propolis ointment appeared to have the most inhibitory effect compared to 15% propolis ointment. This is in accordance with the ointment inhibition zone test in Table 5, where 10% propolis had the most antibacterial effect, especially against *S. aureus* bacteria. Thus, it can be seen that ointment containing 10% propolis extract has an antibacterial effect on secondary infections of scabies, especially on *S. aureus* bacteria. Meanwhile, further research is needed on other types of propolis ointment formulations that are more effective against *S. mutans* bacteria.

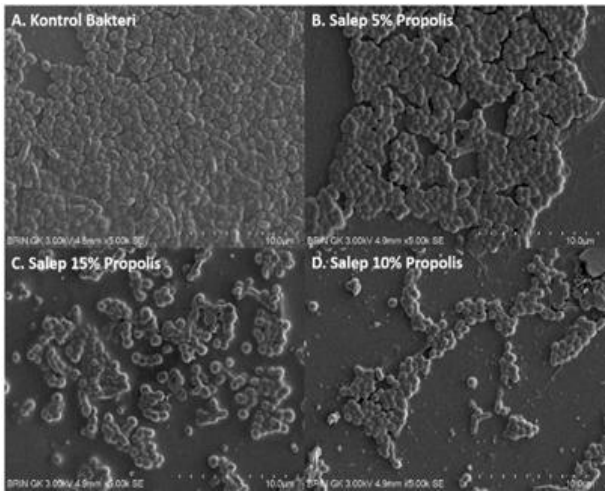

Figure 7. Inhibition of test bacteria mixed culture of *S. mutans* and *S. aureus* by propolis extract ointment using an electron microscope at 5,000x magnification

Conclusion

Based on the research conducted, it can be concluded that propolis extracted with water (distilled water) is more effective in inhibiting and killing *S. mutans* and *S. aureus* bacteria, which cause secondary bacterial infections in scabies inflammation, compared to ethanol propolis extract. A 10% distilled water propolis ointment effectively inhibits the growth of *S. aureus* bacteria but is less effective in inhibiting *S. mutans*.

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