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Antioxidant and Antibacterial Activity of Various Fractions of *Heterotrigona itama* Propolis Found in Kutai Kartanegara

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Abstract

BACKGROUND: In the current pandemic era, people are trying to find additional income, such as stingless bee cultivation. Especially bee species of Heterotrigona itama, because the selling value of stingless bee honey for maintaining health is quite high. However, the potential of other bee products such as propolis is still underutilized.

AIM: In this study, the antioxidant and antibacterial activity of propolis from various fractions were investigated.

MATERIALS AND METHODS: *H. itama* propolis was extracted with 96% ethanol to obtain ethanol extract propolis (EEP). Then, it was used for liquid-liquid partition with different polarities (n-hexane and ethyl acetate) to obtain the n-hexane fraction (HF) and ethyl acetate fraction (EAF). These fractions were tested for antioxidants using the DPPH method and antibacterial against bacteria *Propionibacterium acne*, *Staphylococcus aureus*, and *Escherichia coli* using the agar well diffusion method. Vitamin C was used as a positive control in the antioxidant assay and Thiampenicol was used in the bacterial assay.

RESULTS: The EAF had better antioxidant activity (IC_{50} 128.46 ug/mL) than the ethanol extract (IC_{50} 205.86 ug/mL) and n-HF (IC_{50} 350.01 g/mL). Antibacterial activity of EEP at 200 ug/mL against *P. acne* was 6 ± 1.5 mm, which was categorized as medium inhibition, while the other fractions were classified as weak.

CONCLUSION: EAF had the highest antioxidant activity, meanwhile EEP is the most potent on antibacterial activity. The results obtained are influenced by the environment, where the sample is taken, which is less varied in plant sources and the time of sample collection.

Introduction

Corona disease (Covid-19) is a disease caused by the SARS-CoV-2 virus and was declared a pandemic on March 12, 2020 (Susilo *et a*l., 2020) [1]. At present, COVID-19 is the main focus in the world due to the increasing spread of the disease accompanied by the addition of cases that are still increasing, including in Indonesia (Vollono *et al*., 2020) [1]. Globally, according to the World Health Organization (2021) [2] report, the total confirmed cases of COVID-19 as of January 26, 2021, there were 99,363,697 cases with 2,135,959 deaths (CFR 2.1%) in 223 infected countries and 183 local transmission countries. Indonesia is the highest infected country in ASEAN that has positively confirmed cases of Covid-19(Kemenkes, 2021) [3]. Coronavirus produces free radicals that are uncontrollable and affect the immune system. Free radicals in the body cause there to be large amounts of energy that cause metabolic damage in the body. High levels of free radicals in the body are caused by low levels of antioxidants (Sari *et al*., 2016) [4]. Bacterial coinfection was also found to trigger complications in COVID-19 patients (Prasetyoputri, 2021) [5].

The COVID-19 pandemic has made people look for additional income, one of which is by cultivating stingless bees, especially the *Heterotrigona itama* species. One location where this type of bee was cultivated is in the area near the PLTGU (Gas and Steam Power Plant) in Kutai Kartanegara. Besides honey, *H. itama* bees also produce propolis. Propolis is a product of bees that are used for the manufacture and maintenance of their hives. The production of propolis in stingless bees is very abundant, but there is still limited information about the health benefits of propolis. Propolis is a natural ingredient produced by bees empirically used by the community as an immunomodulator (Sforcin, 2007) [6]. The type of propolis content is influenced by the bee species and resins that vary from the source of the vegetation. Propolis is derived from a mixture of the β-glycosidase enzyme from bee saliva with various plant exudates collected by bees and beeswax. Propolis is very important for bees for hive construction that is waterproof and serves to inhibit fungi, bacteria, and viruses that can damage their habitat. Propolis is a very complex mixture, containing over 300 chemicals, including phenolic compounds, flavonoids, terpenes, and several specific antioxidant compounds (Bachevski *et al*., 2020) [7].

Trigona spp. is one of stingless bee species in Indonesia. To date, there are more than 500 known species of stingless bees, of which about 40 species have potential as honey producers (Michener, 2013) [8]. Differences in the composition of chemical compounds

in propolis greatly affect its biological activity. Mulyati *et al*., 2011 [9] stated that the flavonoid content in propolis produced by *Trigona* spp. was higher than *Apis* spp. In East Kalimantan, the *H. itama* species is one of the species that is widely cultivated by the community (Syafrizal *et al*., 2020) [10].

One of the efforts to prevent disease is to maintain balanced nutrition and consume natural ingredients that have potential as antioxidant. Indonesia, especially in East Kalimantan, has various types of natural resources that have bioactivity. The purpose of this study was to determine the potential antioxidant and antibacterial activity of propolis produced by *H. itama* using two solvents with different polarity levels. In addition, this also want to know that sampling in the PLTGU area affects the bioactivity and class of compounds contained in the propolis. The research was conducted using the DPPH method for antioxidant activity and the agar well diffusion method for antibacterial assay. The bacteria tested were *Staphylococcus aureus*, *Escherichia coli*, and *Propionibacterium acne*. The results of this study are expected to be a reference for scientific evidence regarding the potential biological activity of propolis from *H. itama* stingless bee for the community and breeders in the Kutai Kartanegara PLTGU area.

Materials and Methods

Sample preparation

The main ingredient that will be used in this research is propolis from the *Heterotrigona itama* stingless bees obtained from the PLTGU area in Kutai Kartanegara. Propolis was collected in February 2021. Bee specimen was identified and confirmed at Laboratory of Forest Protection, Faculty of Forestry, Mulawarman University, Samarinda, Indonesia (Identification No. 02/SL-Perlintan/Kht-UM/2021).

Extraction and fractionation of H. itama propolis

Propolis from *H. itama* species was collected from the PLTGU area in Kutai Kartanegara. Dried propolis was put into a maceration vessel then poured 1 L of 96% ethanol, covered, and left for 24 h while stirring occasionally. After 24 h, the macerate was filtered and evaporated over a water bath to obtain an ethanolic extract of propolis (EEP). Then, ethanol extract of propolis was fractionated using two solvents, ethyl acetate which is relatively polar, and n-hexane which is non-polar.

Fractionation was carried out using 20 g EEP, and 400 ml of hot water. Stir the mixture put it in a separating funnel and add 400 ml of ethyl acetate (for the polar fraction) or n-hexane (for the nonpolar fraction) solvent (ratio 1:1). The solvent was mixed then allowed to stand until separate or form two phases. Ethyl acetate and n-hexane were added twice by fractionation until a clear ethyl acetate/n-hexane fraction (HF) was observed; then, it was concentrated to obtain ethyl acetate fraction (EAF) and n-HF.

Antioxidant assay

Antioxidant activity was carried out using the DPPH method (Hairunisa *et al*., 2021) [11], with modification. Samples were dissolved with serial concentrations of 0, 5, 10, 20, 40, 100, and 200 ppm. Add 4.5 mL of each sample and add 1.5 mL of 0.2 mM DPPH. Then incubated at 37°C. After incubation, the solution was put into a cuvette and the absorbance was read in a spectrophotometer at the maximum of 517 nm. Antioxidant activity was obtained using the following equation:

$$\% \text{ Antioxidant activity} = (A0-A1)/A0\times100\%$$

Description: A0: Absorbance control; A1: Sample absorbance

Antibacterial activity

The antibacterial test used Mueller–Hinton agar medium which was poured into a Petri dish and then allowed to solidify. *S. aureus, P. acne*, and *E. coli* bacteria were each inoculated into the medium by diluting the bacteria in a test tube containing 5 mL; then, the suspension was inoculated into the medium and leveled with a hockey stick so that it spread evenly on the surface of the agar medium. Wells were made on an agar medium that had been inoculated with bacteria. In the well, samples of the EAF and n-hexane of stingless bee propolis were added with various concentrations and then incubated for 24 h at 37°C. After 24 h, the clear zone around the well was observed and measured. Thiamphenicol was used as a positive control.

Results

Extraction and fractionation results

The extraction results from the maceration method using 96% ethanol as a solvent obtained a light brown viscous extract with a distinctive odor. The yield obtained is 30% from the 96% ethanol extract, the yield of EAF was 40%, and the n-HF was 38%.

Antioxidant activity

The DPPH method was used to test the EEP, HF, and EAF of *H. itama* propolis from Kutai

Kartanegara. Ascorbic acid (Vitamin C) was used as a positive control. The test results are shown in Figure 1.

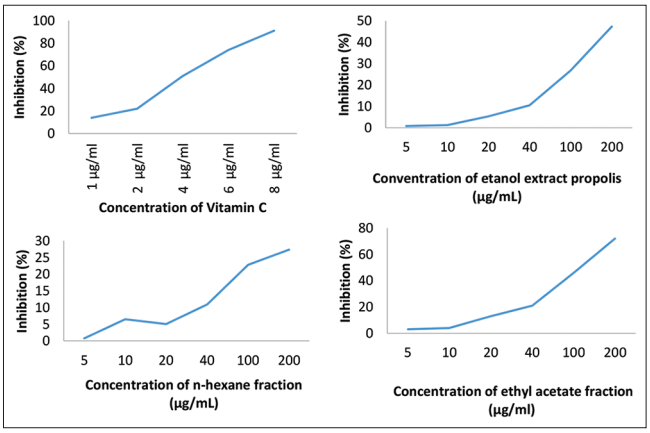

Figure 1: Dose-response curves of antioxidant activity of Vitamin C, ethanol extract propolis, n-hexane fraction, and ethyl acetate fraction of Heterotrigona itama propolis

From the inhibition curve, we can determine the IC_{50} of the extract and the propolis fraction (Table 1).

Table 1: Antioxidant activity (IC_{50}) of *Heterotrigona itama*. propolis extract and fraction

Sample	Equation	IC_{50} (µg/mL)
Vitamin C (Positive control)	y = 20.6x–11.4 R^2 = 0.9784	2.98
EEP	y = 0.2419x+0.2007 R^2 = 0.9939	205.86
HF	y = 0.1314x+4.0088 R^2 = 0.8886	350.01
EAF	y = 0.3562x+4.2397 R^2 = 0.9779	128.46

Antibacterial activity

Antibacterial activity was determined by agar well diffusion method. The bacteria used in this assay were *S. aureus, P. acne,* and *E. coli* bacteria. At first, the fraction was dissolved with acetone, then made four concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml, and 200 µg/ml. Acetone was used as a negative control because it was able to seep into the bacterial epithelium quickly without damaging the bacterial cells (Nuria, 2015) [12]. Then, Thiamphenicol was used as positive control with 30 g/ml concentration. Thiamphenicol was used because it works as an antibiotic with a broad spectrum that can withstand gram-positive and gram-negative bacteria. Then, the resistance was measured using a caliper and analyzed using the SPSS application using the one-way ANOVA method. The results of the inhibition of these bacteria after being given the extract and fraction of *H. itama* propolis are shown in Table 2.

Table 2: Antibacterial activity of *Heterotrigona itama* propolis extract and fraction from Kutai Kartanegara

Sample	Inhibition zone of *Staphylococcus aureus* (mm)				Inhibition
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	categorized
EEP	3	3	4	4	Weak
HF	1	1	1	3	Weak
EAF	4	4,3	4.3	4.3	Weak
Thiamphenicol (30 ug/ml)	20.6				Strong
Sample	Inhibition zone of *Propionibacterium acne* (mm)				Inhibition
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	categorized
EEP	4	4	6	6	Medium
HF	2	2	2	3	Weak
EAF	5	5	5	7	Medium
Thiamphenicol (30 ug/ml)	21				Strong
Sample	Inhibition zone of *Escherichia coli* (mm)				Inhibition
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	categorized
EEP	2	3	3	4	Weak
HF	1	1	2	2	Weak
EAF	4	3	4	4	Weak
Thiamphenicol (30 ug/ml)	21				Strong

Discussion

Based on the results of the research conducted, there was an increase in the antioxidant activity (IC_{50}) of the EEP to the EAF of the stingless bee propolis *H. itama*. The IC_{50} value of the EEP was 205.86 ug/mL and the IC_{50} value of the EAF sample was 128.46 ug/mL. The EEP had very weak antioxidant activity and the EAF sample had moderate antioxidant content based on the Molyneux (2004) [13] category. The increase in antioxidant activity in the EAF was correlated with a higher total phenolic content than the EEP. Although the flavonoid group was not found in the EAF, its antioxidant activity was still better than HF (Yuliawan *et al*., 2021) [14]. The group of compounds that play a role in the EAF is the phenolic compounds. This is probably due to the environment around the PLTGU factory, which is the location of the *H. itama* beekeeping, which still has a little land cover as trees. Several other factors can affect the compound content of propolis, such as the type of tree source of sap, climate zone (time of sample collection), temperature, area, and conditions around the beekeeping (Martysiak-Żurowska and Wenta, 2012) [15]. To date, over 300 compounds have been isolated from propolis and dominated by polyphenol compounds, especially flavonoids, and ester groups (Anjum *et al*., 2019) [16]. The active compounds in propolis vary depending on the plants around the beehive (Costa *et al*., 2020) [17].

Likewise, the antibacterial activity, based on the results of the study of three types of bacteria tested, at a concentration of 200 g/mL, the EAF had better inhibition of *P. acne* bacteria (7 mm ± 1.5 mm) compared to other fractions with medium inhibition category (Surdjowardjojo *et al*., 2015) [18]. After that, the normality test was carried out using the Kolmogorov–Smirnov method, followed by the ANOVA test, to see a significant difference in the bacterial inhibition zone of the six samples on bacterial growth. A significant difference was found between the results of the inhibitory fraction and the positive control of Thiamphenicol.

Antibacterial activity is also influenced by the class of compounds present in the fraction (Soroy *et al*., 2014; Pavlovic *et al*., 2020; Zaccaria

et al., 2019) [19], [20], [21]. The EAF has a class of non-flavonoid phenolic compounds that affect its antioxidant and antibacterial activity.

Conclusion

From the results of the research that has been done, it can be concluded that:

1. The antioxidant activity of the EAF (IC_{50} 128.46 g/mL) was higher than that of the HF (IC_{50} 350.01 μg/mL). This is proportional to the phenolic content of the fraction
2. The antibacterial activity of the fraction against 3 types of bacteria showed a weak inhibitory category compared to the positive control (Thiamphenicol). At a concentration of 200 g/mL, EAF had medium (7 ± 1.5 mm) inhibition on *P. acne* bacteria compared with other fractions.

Future Perspective

Given the result of antioxidant activity of *H. itama* propolis extract and its fraction, several pharmacological assays can be carried out for the future perspective. Anti-inflammatory and anticancer activities are the most feasible assay that corresponds to the result that we showed in this study.

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