Journal of
Fundamental and Applied Pharmaceutical Science
https://journal.umy.ac.id/index.php/jfap

Identification of Secondary Metabolites and Antibacterial Activity of Non Polar Fraction from *Heterotrigona itama* Propolis

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Abstract

Propolis is one of the natural products produced by kelulut bees and is still not widely used. The type of stingless bee that is the prima donna in the community is *Heterotrigona itama*. This study aims to determine the phytochemical content of the n-hexane fraction of *Heterotrigona itama* bee propolis collected from Kutai Kartanegara, East Kalimantan. The n-hexane fraction was obtained from the methanol extract of H. itama propolis by the liquid-liquid partition method. After obtaining the n-hexane fraction, the research continued with a qualitative phytochemical test to identify the compound and total phenolic content. Antibacterial activity was determined by the agar well diffusion method with a serial concentration in *Escherichia coli* bacteria. Qualitative phytochemical analysis in the form of color changes showed that the n-hexane fraction of H. itama propolis contained flavonoids, alkaloids, saponins, and tannins. Based on the results, the total phenolic content of the n-hexane fraction sample was 490 mgGAE/100 g. It caused the n-hexane fraction to have lower phenolic content than the methanol extract (792 mg GAE100 g). Furthermore, this result indicated that the non-polar fraction was not substantial enough to extracted phenolic compounds. It correlated to the antibacterial activity of the n-hexane fraction, which was very weak (2 mm ± 1.5) at 200µg/mL concentration.

Keywords: *heterotrigona itama*; n-hexane; phenolic content; propolis

Data of article
Received : 30 July 2021
Reviewed : 04 Aug 2021
Accepted : 24 Aug 2021

DOI
10.18196/jfaps.v2i1.12406

Type of article:
Research

INTRODUCTION

Indonesia has incredible biodiversity, but non-timber forest products' potential is still limited and not managed properly. Bee product is one of the non-timber forest products that have a potential effect on health. Nowadays, in the pandemic era, stingless bee (*kelulut* bee) products have more attention for maintaining our health than utilized as a biodiversity-based economic potential[1]. This honey is even more expensive than honey from *Apis* spp bees. However, the potential use of another product, such as propolis, is still limited. If this potential is appropriately managed, it can contribute to the community's economy, especially the villagers. According to FAO (Food and Agriculture Organization), beekeeping is

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one of the best economic opportunities for people living in forest areas[2]. These benefits are directly obtained in the form of beekeeping products. Generally, propolis was generated by different resinous substances with antimicrobial activities from plants near the hive and collected to protect the hive. Bee colonies make a barrier immunity in the hive against pathogens and parasites[3], while propolis has prospective as a natural antibacterial.

Escherichia coli, one of the anaerobic facultative gram-negative bacteria, was abnormal flora in the large intestine of humans. Most of the strains of this bacterium are harmless, but some strains have acquired the DNA encoding for plasmid bacteriophage or enterotoxin or invasion factor and become pathogenic. Strains that contain this virulence factor are responsible for the incidence of global diarrhea, neonatal meningitis, sepsis, and urinary tract infection[4]. If the bacteria are located outside the intestine or in their abnormal habitat, *E. coli* can become pathogenic bacteria. Furthermore, *E. coli* with certain strains usually cause acute diarrhea. This bacteria can be transmitted through water, food, or people who have bad sanitation[5,6]. To overcome this, natural ingredients are used, such as propolis which is usually naturally used by bees to protect their hives from bacteria and fungi.

Heterotrigona itama is the most cultivated species of *kelulut* bee in Indonesia. In East Kalimantan, people are starting to be interested in cultivating *kelulut* bees. The community around *PLTGU (Pembangkit Listrik Tenaga Gas dan Uap)* in Kutai Kartanegara region try to develop *kelulut* bee farms as additional income in this pandemic era. Previous research showed that the ethyl acetate fraction of propolis from that location contains an alkaloid, saponin, and terpenoid[7]. This study was conducted to identify the secondary metabolite of n-hexane fraction and antibacterial activity against *E. coli*. The n-hexane fraction from methanolic extract of plants source indicated has inhibition to multidrug resistance human pathogenic strains[8,9,10]. Propolis is collected by a bee from plant resin to prevent bacteria and fungi from their hive. Based on the background above, this study aims to evaluate the antibacterial activity of the n-hexane fraction of stingless bee propolis from that area.

METHOD

This study used *Heterotrigona itama* propolis obtained from Bukit Biru, Kutai Kartanegara, East Kalimantan. Furthermore, the used materials also included methanol, Folin Ciocalteu reagent (Merck), sodium carbonate (Na_2CO_3) (Merck), n-hexane ($CH_3(CH_2)_4CH_3$), distilled water, gallic acid (Sigma-Aldrich), ethanol pro analysis (Merck), 70% ethanol (CV. Mulawarman Medika), filter paper, Quercetin (Sigma Aldrich), aqua dest (CV. Mulawarman Medika), and aluminum foil. The Genesys 10S UV-Vis Spectrophotometer was used as instrument analysis.

Sample preparation

Fresh propolis was collected from *kelulut* bee apiary, then kept at -4°C until used. The dirt, bees, and larva were removed out from propolis, then cut into pieces. The 100 grams of propolis were kept in containers, and methanol solvent was added and mixed. The Propolis mixture that has been left for 24 hours was filtered, and the solvent was changed 3 times. The filtrate was evaporated at low pressure at a temperature of 60-70°C using an evaporator to obtain a thick extract[11].

Specimen of stingless bee was determined at Laboratory of Faculty of Forestry, Mulawarman University and identify as *H. Itama*.

Fractionation

The 20 g of methanol extract was put into a container then added 400 ml of hot water. The extract was mixed, added in a separating funnel, and 400 ml of n-hexane solvent was added in a ratio (1:1). It was mixed by shaking for one minute then allowed to stand until the solution separated and formed two layers. Furthermore, n-hexane fraction filtrate was obtained and evaporated until a thick extract was obtained, and the yield was calculated.

Phytochemical Assay

Flavonoid Test Identification

The extract was pipetted as much as three drops into the drip plate. The extract was then added with 1 drop of H_2SO_4 p.a. Positive samples contain flavonoids if the solution undergoes a very striking color change to yellow, red, or brown.[12]

Tannin Test Identification

The 1 mL methanol extract of propolis was added into a test tube and added 2-3 drops of 1% $FeCl_3$. Samples were contained tannins when there was a color change from light green to blackish green.[13]

Alkaloid Test Identification

A sample of 1 ml was put in a test tube, then 1 drop of HCl was added and homogenized. Next, 3 drops of Meyer's reagent were added. The test results were considered positive for containing alkaloids if the precipitate was yellowish-white.[14]

Terpenoid Test Identification

The 1 ml of ethyl acetate fraction was taken and put into a test tube, and 0.5 ml of chloroform was added. A few drops of $H2SO4$ concentrated were later added to the side of the tube. If it showed reddish-brown color between the surfaces, it indicated the presence of triterpenoid compounds.[14]

Saponins Test Identification

A sample of 1 ml was put in a test tube. The 1 ml of distilled water was added, then shake, and let stand for 30 seconds. Furthermore, the 5 drops of 1% HCl were mixed into the solution. The presence of saponin compounded with the formation of foam.[14]

Total Phenolic Content Assay

First, The 10 % Na_2 CO_3 solution was prepared 24 hours before use. On the next day, the n-hexane fraction of propolis was dissolved with 10 % methanol and distilled water to make a 10 mg/mL concentration. The gallic acid was used as a standard curve with a 3-50 µg/mL concentration. The phenolic content of the standard curve was determined with a 10 mL solution of 500 µL gallic acid, 500 µL of Folin-Ciocalteureagent, 3 mL of 10% $Na2CO3$ solution distilled water. It was then incubated for 2 hours in a dark condition and measured on a UV-Vis spectrophotometer at a wavelength of 765 nm. The total phenolic content was calculated as natural compound (gallic acid) equivalent (GAE) by the following equation: T= C XV/M. T is the total phenolic content in mg/g of the extracts as GAE, while C is the concentration of gallic acid established from the calibration curve in mg/ml. V is the volume of the extract solution in ml, and M is the weight of the extract in g. The total phenolic content analysis results were expressed in units of g Gallic Acid Equivalent (GAE)/

100g samples. The n-hexane fraction was carried out in the same step of total phenolic determination.[15]

Antibacterial Assay

The antibacterial test used the agar well disk of diffusion method with some modification[16]. First, the media agar was prepared to culture bacteria and sterilized, then 20mL Nutrient Agar solution was poured into Petri dishes and wait until solid. After that, the 20 µL *E.coli* bacteria solution was added and spread on the surface of the media on the plates. Media agar plates were divided into five holes with a size four-mm diameter. The 20 µL of 25-200 µg extracts in acetone solution was added into the hole. A concentration of 30 µg/20 µL of positive control (Chloramphenicol) was added to each Petri disk. Acetone was used as a negative control and then incubated in the dark at 32°C for 24 h. Inhibition of bacteria was measured (mm) by calipers. Antibacterial activity was calculated as the mean inhibition zone for the test sample divided by the mean inhibition zone for the standard drug.

RESULTS AND DISCUSSION

Phytochemical Screening

The phytochemical screening process is one of the preliminary stages in providing an overview of the class of compounds contained in *H. itama* propolis to overview the sample. The results of phytochemical screening of stingless bee propolis extract contained secondary metabolite compounds, which can be seen in Table 1.

Table 1. Phytochemical Screening of n-Hexane Fraction from *Heterotrigona itama* Propolis

Compounds	Result	Indicator
Flavonoid	+	Color change to yellow, red to brown
Alkaloid	+	Yellowish-white precipitate
Terpenoid	-	No color change between the surfaces
Tanin	+	Color change from light green to blackish green
Saponin	+	Changes in the initial solution and showed foam

Based on the data in Table 1, stingless bee propolis extract contains flavonoids, alkaloids, tannins, and saponins. These compounds are secondary metabolites produced from plants. This condition is related to the food source of the bees[17,18]. Stingless bees visit the several flowers of plants for nectar and pollen[19]. In addition, stingless bees take plant sap to build their nests[20,21,22]. The n-hexane fraction was obtained for the phenolic determination. Compounds of phenol are described by the presence of a light green or slightly blue color, and the sample shows a blackish green stain so that it is suspected to be positive for phenolic[23]. Phenolic responds with 1% $FeCl_3$ to frame intense red, blue, purple, or dark colors as $FeCl_3$ will respond with –OH. Aromatics identification of tannins can also be carried out with ferric chloride expansion. One of them is because tannins are polyphenolic compounds, which means they have a place with a group of phenolic compounds. Both examples show positive tannins by showing a blackish green stain[24].

Furthermore, a few drops of concentrated HCl and magnesium were added to the sample during the flavonoid test. Flavonoids were indicated by the appearance of maroon or pink color within 3 minutes. Both samples showed a red

color suspected to be positive for flavonoids. Besides, in the saponin test, distilled water is added to the sample, then bubbled using a water shower and shaken to form a stable foam. A fixed foam formation indicates the presence of saponins. Two examples showed positive results. Saponin compounds have a glycosyl group as a polar group and a steroid or triterpenoid group as a nonpolar group. Thus, they surfaced dynamically and formed micelles when it is shaken with water. In micellar structure, the polar groups face outward while the nonpolar groups face inwards, which looks like foam[25].

Total phenolic content

The total phenolic content results showed phenolic compounds from the n-hexane fraction of *H. itama* propolis with absorbance values (Table 2). The data generated from the total phenolic test content was processed in absorbance tables and standard curves (Figure 1).

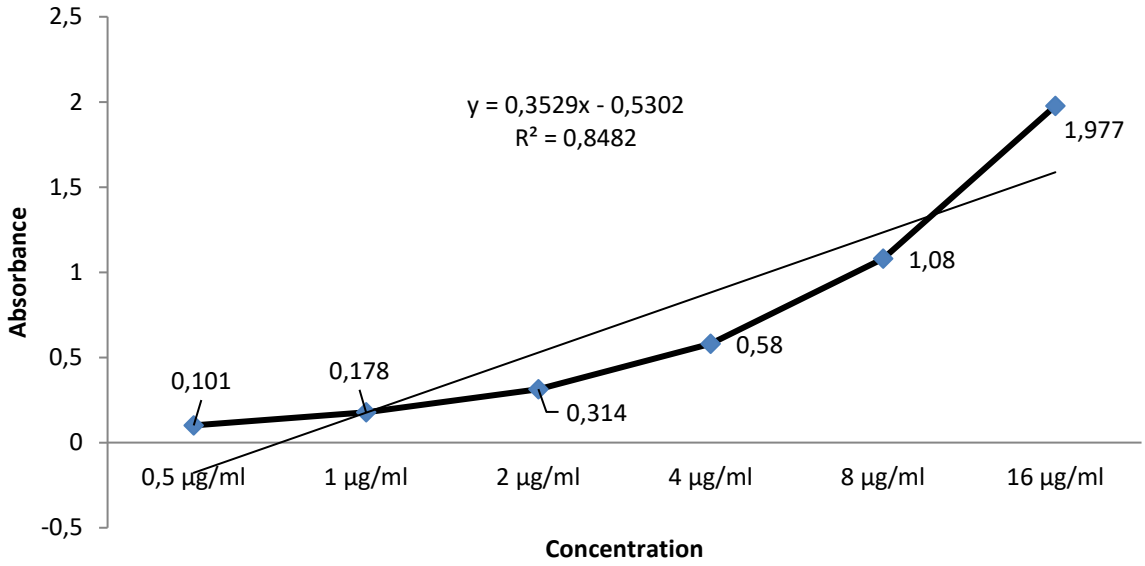

Figure1. Standard curve of gallic acid to determine phenolic content

Furthermore, the researchers calculated the total phenolic content of the n-hexane fraction of propolis with gallic acid equivalent in Table 2.

Table 2. Total phenolic content of n-hexane fraction from *H. Itama* propolis.

Absorbance	Mean of Absorbance	Gallic Acid Equivalent (µgGAE/mL)	Total Phenolic Content (mgGAE /100 g)
0.896 0.889	0.3375	2.45	490

The results of the measurement of the phenolic content of propolis extract depend on the solvent as the solvent can affect the total phenolic content. When mixed in food, the phenolic compounds contained in propolis extract will be absorbed. However, it does not change in structure to increase the function of the food as an antioxidant. The phenol content of the *kelulut* bee *H.itama* propolis was determined by UV-Vis spectrophotometry, with the standard used gallic acid as gallic acid is a derivative of hydroxyl benzoic acid indicating a simple phenolic acid. The researchers used gallic acid as a standard based on the

availability of a stable and pure substance. Another factor is that gallic acid is cheaper than other standard compounds. The total phenol content was calculated by putting the sample absorption value at a wavelength of 767 nm into the linear equation y= ax+b obtained from the gallic acid calibration curve. The yield is expressed in mg gallic acid equivalent per 100 grams(mg GAE/100 grams). Standard curves were made with a concentration of 0.5; 1; 2; 4; 8; 16 g/mL. The calibration curve was made from the level of gallic acid done in duplicate, and the R2 value was searched. The R2 or coefficient of determination value is a number whose value ranges from 0 to 1, indicating how close the estimated value for regression analysis represents the actual data. Regression analysis will be the closest and most reliable one if the R2 value is equal to or close to 1. Based on the calibration curve measurement results, the R2 value was 0.8482. The tested phenolic content had the highest concentration in the n-hexane fraction, which was 490 mgGAE/g, while the phenolic content in the methanol extract was 792 mgGAE/g. The total phenolic content test results were influenced by the high methanol content and could also affect the withdrawal process of phenolic compounds. It means the higher the methanol content is, the more the absorbed phenolic compounds will be. It occurs because *H.itama* propolis has a short hydrocarbon chain so that the non-polar solvent ability of n-hexane in absorbing extracts from propolis is still lacking. Based on the total phenolic test results, the total phenolic content in the n-hexane fraction sample was lower than the methanol extract (Table 3.)

Table 3. Comparison of total phenolic content

Sample	Total Phenolic Content
n-Hexane Fraction	490 mgGAE/100 g
Methanol Extract	792 mgGAE/100 g

This result aligns with the research of[26,27], stating that the total phenolic content of methanol extract was more significant than the n-hexane fraction. It could be due to the polar nature of methanol, which could attract phenolic compounds well in the extraction process. Moreover, it also aligns with the results of previous studies revealing that methanol was a polar solvent that did not have a high boiling point so that compounds susceptible to high temperatures would not be damaged.[28] Furthermore, the chemical composition of propolis varies qualitatively and quantitatively due to the diversity of plant resins, in addition to the various geographic and climatic characteristics of the environment around the beehive.[29,30]

Antibacterial activity

Antibacterial activity of non-polar fraction from *H. itama* propolis was determined with agar well diffusion. Acetone was used as a negative control, and broad-spectrum antibacterial drug (Chloramphenicol) was used as a positive control. *E. coli* is a gram-negative bacterium and is well known for its resistance to many antibiotics due to the permeability barrier provided by its outer membrane[31]. The result of antibacterial activity of n-hexane fraction of *H. itama* propolis against *E. Coli* showed a weak level of inhibition (Table 4).

Table 4. Antibacterial activity of n-hexane fraction of *H. itama* propolis against *E. Coli*

Concentration (µg/mL)	Inhibition zone (mm)				Antibacterial Category
	Rep. 1	Rep. 2	Rep. 3	Mean ± SD	
25	1	0	1	1 ± 0.5	weak
50	3	1	1	1 ± 1.1	weak
100	3	2	1	2 ± 1	weak
200	3	4	1	2 ± 1.5	weak
Chloramphenicol	21	20	17	19 ± 2	very strong
Control (-)	-	-	-	-	

The antibacterial activity was categorized by diameter inhibition. If the inhibition diameter regions on 5 mm or less, antibacterial activities are categorized as weak. 5-10 mm is categorized as medium; 10-19 mm is categorized as strong, while 20 mm or more is categorized as very strong[32]. The maximum concentration of 200 µg/mL only showed inhibition of 2 mm width. Table 4 shows the inhibition in the weak category as it is around 1-2 mm. The non-polar fraction of propolis was had weak activity against *E.coli.* This gram-negative bacteria activity was caused by differences in the structure of the outer membrane of bacteria and the production of hydrolytic enzymes that caused degradation of the active components of propolis[33]. Most of the polyphenol compound was detected in polar solvent rather than non-polar solvent[34]. Propolis was collected by a bee from plant resin and correlated to a specific compound from that plant. Bioactivities of plants were related to total phenolic compounds, such as flavonoids and lignin as an antioxidant, alkaloid as antibacteria[35].

The low content of phenolic components in the n-hexane fraction of *H.itama* propolis was correlated with antibacterial activity against *E.coli*. Another research about the antibacterial activity of the n-hexane fraction of plant extract showed relatively low phenolic content.[36] The n-hexane fraction also indicates it had good gram-positive activity rather than gram-negative bacteria[37]. That present of secondary metabolite in n-hexane fraction and phenolic content also had a contribution to antibacterial activity. It is was influenced by the time of sample collected, climate, and plants near the sample collected[38]. The chemical composition of propolis was determined by the composition of the plant source[39]. Most of the activity was not directly affected by the presence of PLTGU

CONCLUSION

The n-hexane fraction of *H. itama* propolis contained alkaloid, flavonoid, saponin, and tannin, indicating potential compounds. This non-polar fraction was insufficient to extract phenolic compounds (490 mg GAE/100 g). The total phenolic content of the non-polar fraction, which was less than the crude extract, indicated that some phenolic compounds were present in polar fractions. It correlated to the weak antibacterial activity of n-hexane fraction (2 mm ± 1.5) at 200µg/mL concentration. Furthermore, the results of this study are preliminary studies. Further research is suggested to carry out the bioactivity of the *kelulut* bee propolis H.itama from Kutai Kartanegara to benefit the community for the use of *kelulut* bee propolis.

ACKNOWLEDGMENT

The authors would like to thank the research institute and community service

Universitas Muhammadiyah Kalimantan Timur (KDM grant) for their support in this study.

CONFLICT OF INTEREST

All authors declare no potential for conflict of interest with the research, authorship, and article publication.

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