ORIGINAL ARTICLE

Comparative Analysis of Phytochemical, Total Phenolic Content, Antioxidant and Antibacterial Activity of Two Species Stingless Bee Propolis from East Kalimantan

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ABSTRACT

Introduction: *Geniotrigona thoracica* and Heterotrigona itama are the most common stingless bee species found in East Kalimantan. One of the bee products is propolis. However, the utilization of that propolis is still limited. The purpose of this research is to investigate phytochemical, antioxidant and antibacterial potential from *G. thoracica* and *H. itama* propolis. **Methods:** The compound group was identified by phytochemical tests and Folin-Cocalteau's assay was used to determine total phenolic content. DPPH (2,2-diphenyl-1-picryhydra-zyl) assay was used to determine antioxidant activity and agar well diffusion method was used to determined antibacterial activity against Staphylococcus aureus. **Results:** The phytochemical test showed that *G. thoracica* propolis extract contained saponins, flavonoids, terpenoids, and tannins, while the propolis of *H. itama* contained alkaloids, terpenoids, and tannins. The *H. itama* propolis extract showed strong antioxidant efficacy (84%) compared to *G. thoracica* propolis (76,5 %). The total phenolic content of both propolis extract was 875 and 880 mg GAE/100g respectively. Propolis extract from those two species had weak antibacterial activity against S. aureus. **Conclusion:** In accordance with differences in metabolite contain, both propolis extract had potential antioxidant activity, while both were not effective in antibacterial activity against S. aureus. The environment of apiary location was very influential.

Keywords: Antioxidant, Antibacterial, *Geniotrigona thoracica*, *Heterotrigona itama*, East Kalimantan

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INTRODUCTION

Kalimantan is the second largest island in Indonesia which is famous as an area that has a lot of forest. The wilderness in this part of Kalimantan is home to various bees, one of which is the stingless bee. The stingless bee is a honey-producing bee from Meliponidae family and the Trigona genus. Stingless bees produce a substance to protect their hives from harmful environmental threats or attacks by other organisms called Propolis (made from plant resins collected by bees). *Trigona* spp. is able to produce propolis in large quantities as much as 6,7 kg/year (1). *Heterotrigona itama* and *Geniotrigona thoracia* produce more abundant propolis than other types of propolis-producing bees. *Geniotrigona thoracica* is a type of stingless bee that is quite special compared to other stingless bees because it has easily distinguishable characteristics such as its larger size and body color which is dominated by brownish black (2).

Stingless bee propolis has antioxidant activity because the content contained in propolis is able to prevent and repair cells in the body caused by exposure to free radicals (3). Propolis has various pharmacological activities such as antioxidant, antibacterial, anticancer, antifungal, anti-inflammatory, antiviral, and antidiabetic because of its diverse and complex chemical composition (4). Natural antioxidant activity of secondary metabolites in the form of phenols and flavonoids (5).

Antioxidants are substances that at small concentrations are significantly able to inhibit or prevent oxidation of the substrate caused by free radicals (6). Free radicals that are produced continuously during normal metabolic processes are considered to be the cause of damage to the function of body cells which eventually triggers the onset of degenerative diseases (7). The antioxidant activity of a compound can be classified based on the IC_{50} value. If the IC_{50} value of an extract is below 50 ppm then the antioxidant activity is very strong category, the IC_{50} value is between 50-100 ppm meaning the

antioxidant activity is strong, the IC_{50} value is between 100-150 ppm meaning the antioxidant activity is in the moderate category, the IC_{50} value is between 150-200 ppm means the antioxidant activity is weak category, whereas if the IC_{50} value is above 200 ppm then the antioxidant activity is categorized as very weak (8). The role of these antioxidants is very important in the body's defense and recovery processes.

Antibacterial activity in propolis has the advantage of not causing resistance and has small side effects and has high selectivity against pathogenic bacteria (9). Antibacterial are compounds in small concentrations capable of inhibiting or killing harmful microbes (10).

Stingless bees that produce abundant propolis are easy to find in East Kalimantan which has great potential for development, especially *Heterotrigona itama* and *Geniotrigona thoracica*. It is necessary to study the phytochemical, antioxidant and antibacterial activities of propolis, to provide a comparison of its potency. The total phenolic content was also traced as a description of the role of phenolic compounds in this phytochemical analysis. This information can later be used as a species selection reference for beekeepers and researchers in developing propolis bee products in this area.

MATERIALS AND METHODS

Sample preparation

Propolis from two different stingless bee species were collected from bee farms around the Unmul Research Forest or Kebun Raya Unmul Samarinda (KRUS) and bee specimens were taken to identify the correct species. Species identification based on bee morphology and characteristic of nest entrance (Figure 1) was carried out at the Forest Protection Laboratory, Faculty of Forestry, Mulawarman University in April 2021 with identification test number 02/SL-Perlintan/Kht-UM/2021. Based on the identification, it can be ascertained that the propolis samples used in this study were propolis types of *H. itama* and *G. thoracica*.

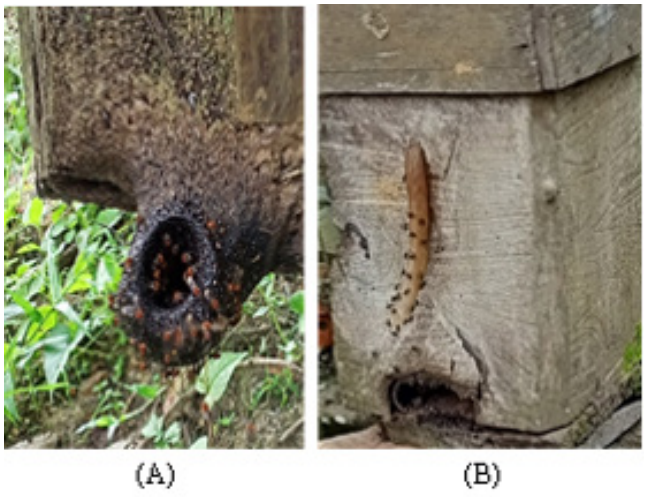

Figure 1: Characteristic of nest entrance (A) Geniotrigona thoracica, (B) Heterotrigona itama

Extraction

Heterotrigona itama and *Geniotrigona thoracica* bee propolis were macerated using methanol and allowed to stand for 24 hours while stirring occasionally. The supernatants were filtered and the leftover residue were re-macerated for another 24 hours and repeat the filtration process. The filtrate was subsequently evaporated using the water bath to obtain methanol extract of propolis. The dried extracts were then weighed to determine the extraction yields.

Phytochemical assay

Phytochemical assays were carried out as previously described (11). The phytochemical assays were conducted to detect the following compounds:

Alkaloids

A total of 5 mL of the extract was added with 2 mL of concentrated hydrochloric acid, followed by the addition of 1 mL of Dragendroff's reagent. The color of the solution will turn red or orange, indicating that the extract is positive for alkaloids.

Flavonoids

A total of 1 mL of the extract was added a few drops of 1% NaOH, the solution had a bright yellow color, then 1% HCl was added. The solution turned colorless, indicating a positive extract contain flavonoids.

Triterpenoid

A total of 1 ml of extract was added 0.5 ml of chloroform. Then a few drops of H2SO4 concentrate was added on the side of the tube. The reddish brown color between the surfaces was indicated the presence of triterpenoid compounds.

Tanin

A total of 2 mL of the extract was then given a few drops of 1% FeCl3 solution. The positive extract contained phenol if the color changed to green.

Coumarin

A total of 1 mL of the extract was then added with 95% ethanol and 1 mL of 1% NaOH solution each. The positive extract solution contains coumarin if there is a change in color to yellow.

Saponins

A total of 2 mL of the extract was put into the tube, then 10 mL of distilled water was added. The solution was shaken for 1 minute. The formation of stable foam indicated that the extract contained saponins.

Total phenolic content

The total phenolic compound was determined using the Folin Ciocalteu method. A series of stock solution of *Heterotrigona itama* and *Geniotrigona thoracica*

propolis extracts were made. Each concentration was then added 1 mL of Folin-Ciocalteu reagent and the solution was allowed to stand. After 3 minutes, 1 mL of 1% sodium carbonate solution was added. Then the solution was added with distilled water up to 10 mL and incubated for 90 minutes. Absorbance measurements were carried out using UV-Vis Spectrophotometry at 725 nm. The results obtained were then calculated using a standard gallic acid calibration curve.

Antioxidant activity

Determination of antioxidant activity (12) was carried out using the DPPH method (2,2-diphenyl-1-picryhydra-zyl). Several series of concentration solutions were made and ascorbic acid was used as a positive control. Then the ascorbic acid solution and the extract solution were added with 3 mL of DPPH solution and methanol up to 10 mL in a volumetric flask. The solution was then incubated for 30 minutes in the dark. Absorbance measurements were carried out using UV-Vis spectrophotometry with a wavelength of 517 nm using methanol as a blank. The test results are used to determine the value of % inhibition.

Inhibition % = [(ABlank-ASample)/ABlank] x 100

Antibacterial activity

The antibacterial activity of Heterotrigona itama and *Geniotrigona thoracica* extracts against *S. aureus* bacteria was carried out using the well method. The bacterial suspension was put into each of 3 petri dishes containing Mueller Hinton Agar (MAH) media. The wells that have been made in each petri dish are divided into 6 parts and the extract with a concentration series of 25, 50, 100, and 200 g/mL is added with positive control and a negative control as a comparison. Then the petri dish was put into the refrigerator to be able to diffuse for 24 hours. Then incubated in an incubator at 37°C for 24 hours. An inhibition zone will be formed and can be measured using a caliper.

RESULTS

Extraction analysis

The propolis extraction process was conducted by the maceration method, which is a process of withdrawing the active compound in the material through immersion using a suitable solvent without heating, so that the thermally unstable compound will not be damaged. In this research, methanol was used as the solvent to extract the relatively polar constituent of the propolis. The methanolic extract yields were 29.44% and 33.96% for *H. itama* and *G. thoracica* propolis samples, respectively (Table I).

Phytochemical and total phenolic content analysis

Phytochemical analysis was conducted qualitatively using various detection reagent. The results of the phytochemical test showed that the *H. itama* propolis extract contains alkaloid, terpenoid, and tannin compounds, while the *G. thoracica* propolis extract showed positive content of saponins, alkaloids, terpenoid, and tannins (Table II).

Table I: Yield of propolis extract

Sample of Propolis	Weight of Sample (g)	Weight of Extract (g)	Yield (%)
H. itama	50	14.72	29.44
G. thoracica	50	16.98	33.96

Analysis of the total phenolic compounds was carried out by the Folin-Ciocalteau method using UV-Vis Spectrophotometry with a wavelength of 767 nm. Gallic acid was used as standard (Figure 2). The total phenolic compound was calculated using the gallic acid standard linear equation to obtain the amount equivalent to gallic acid or gallic acid equivalent (GAE). The total phenolic content in *H. itama* propolis extract was 875 mgGAE/100g, while in *G. thoracica* propolis extract was 880 mgGAE/100g (Figure 3).

Antioxidant analysis

Determination of the antioxidant activity of propolis extracts were carried out using the DPPH method

Table II. Phytochemical comparison of propolis from *H. itama* and *G. thoracica*

Phytochemical content	Propolis sample	
	H. itama	*G. thoracica*
Alkaloid	+	-
Flavonoid	-	+
Terpenoid	+	+
Saponin	-	+
Tannin	+	+
Coumarin	-	-

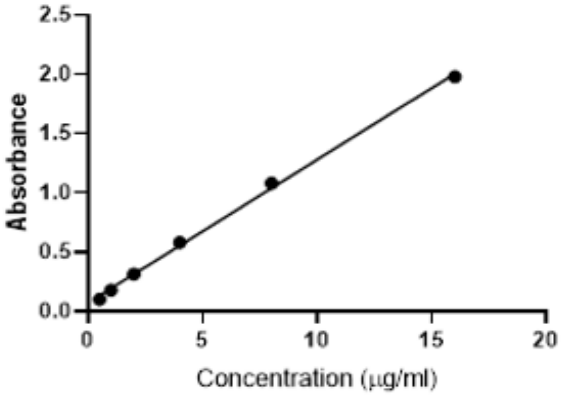

Figure 2: Gallic Acid Standar Calibration Curve

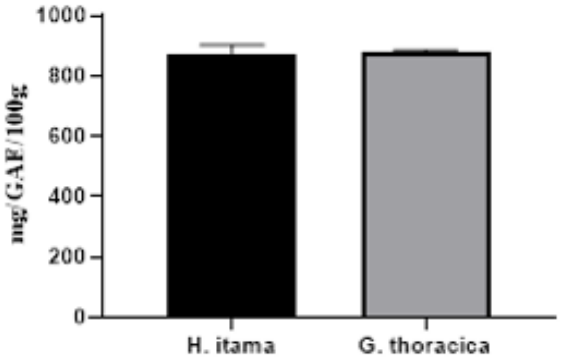

Figure 3: Total phenolic contents (TPC) of methanolic extract of *H. itama* and *G. thoracica* propolis

(Figure 4). The IC_{50} is the concetration in which the samples give 50% radical scavenging activity. The value of IC_{50}indicates the potency of antioxidant activity of propolis as the lowest concentration give the most potency on the activity. The IC_{50} value of methanolic extract of *H. itama* and *G. thoracica* propolis was 50.61 and 42.55 ppm respectively. This showed that *G. thoracica* propolis has slightly higher potency on antioxidant activity. The result also showed that despite has higher antioxidant activity, *G. thoracica* propolis exhibited lower efficacy compared to *H. itama* propolis. The antioxidant activity of *G. thoracica* propolis was saturated at concentration of 50 ppm with 60% inhibition at its maximum activity, meanwhile *H. itama* propolis showed higher efficacy with maximum activity was observed at 200 ppm with 100% inhibition.

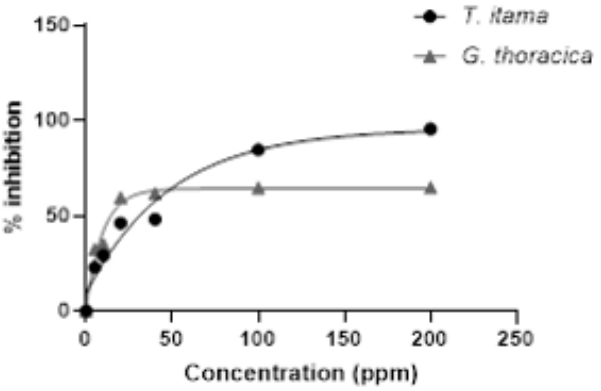

Figure 4: Antioxidant activity of methanolic extract of *H. itama* and *G. thoracica* propolis. The methanolic extract of *H. itama* has stronger antioxidant efficacy but less potency compared to *G. thoracica*.

Antibacterial analysis

The antibacterial activity was evaluated by measuring the inhibition zone of bacterial growth. The result of antibacterial assay from methanolic extract of *H. itama* and *G. thoracica* showed that both extract have range of inhibition at 1-3 mm and is considered as weak activity against S. aureus (Table III). The increase of concentration did not result on significant increase of inhibition zone. Chloramphenicol was used as positive control and showed high inhibition on S. aureus growth.

Table III: Antibacterial activity of the methanolic extract of *H. itama* and *G. thoracica* propolis

Conc. (µg/mL)	Inhibition of sample against *S. aureus* (mm)		
	H. itama	*G. thoracica*	Chloramphenicol (30 ug/mL)
25	1 ± 0.57	1 ± 0.57	21 ± 0.57
50	2 ± 0.75	1 ± 0.57	
100	2 ± 0.84	2 ± 1.01	
200	3 ± 1.15	2 ± 1.01	

DISCUSSION

Propolis is a natural product from bees that is rich in efficacy and has been used for a long time (13). In this study, a comparison of the activity of propolis extracts from two different bees, namely *H. itama* and *G. thoracica* was carried out. *G. thoracica* showed more complex content compare to *H. itama*. *G. thoracica* propolis extract contains flavonoids, terpenoids, saponins and tannins, while *H. itama* propolis only contains alkaloids, terpenoids and tannins. This result is in contrast to the previous study which showed that *H. itama* has more complex content compared to *G. thoracica* (14,15). The difference in the compound content in the two extracts can be caused by differences such as the type of bee food, the location of the nest (16). Polyphenol compounds such as tannins and flavonoids contained in both propolis extracts have the ability to bind free radicals (17). The difference in phytochemical content between these two stingless bee species is also caused by the influence of weather and the tendency of this bee species to choose plants as a source of propolis.

In this study we observed that the total phenolic content of methanolic extract of *H. itama* propolis is similar to *G. thoracica*. The total phenolic content showed the different results when compared with previous studies which obtained 56.90 g/mL in *H. itama* propolis extract and 29.10 g/mL in *G. thoracica* (18) which indicated that the *H. itama* propolis has higher level of total phenolic content compare to the *G. thoracica* propolis. The difference in these results can be caused by differences in the solvent used and the area where the propolis originated (19).

The DPPH method was used to determine the ability of propolis extract to bind free radicals and subsequently evaluate the IC50 value, the concentration of the extract to be able to inhibit free radicals by 50% (20). In this study we found the IC50 value were 50.61 and 42.55 ppm for *H. itama* and *G. thoracica* respectively. The value obtained is greater than the IC50 of ascorbic acid, which is 2.9 ppm. The IC50 value of the two propolis extracts is categorised as very strong level of antioxidant activity (21), although not as strong as ascorbic acid. The antioxidant potency of ethanolic extract of *G. thoracica*

is slightly better compare to *H. itama* propolis. On the other hand, the efficacy of *H. itama* propolis (84% inhibition) was higher compare to *G. thoracica* (76.5 % inhibition) at 100 ppm. The antioxidant activity of *G. thoracica* propolis was saturated at concentration of 50 ppm, this effect might be due to the presence of the ballast substance in methanolic extract of *G. thoracica* propolis. The ballast substance can interfere the antioxidant activity and consequently an increase in concentration is not followed by an increase in effect. In contrast to this finding, previous studies reported that ethanolic extract of *H. itama* has greater antioxidant potency compare to *G. thoracica* propolis (18,22). Based on phytochemical analysis, the presence of flavonoid may contribute to better antioxidant potency of *G. thoracica*. Together, the results suggest that solvent and the location where the propolis originated, play role on antioxidant activity of the propolis. The antioxidant effect was associated with cancer prevention activity. The reactive oxygen species play important role on DNA damage that lead to mutagenesis and carcinogenesis. The antioxidant activity may intercept the free radical activity and prevent the DNA damage process. The presence of flavonoid in propolis suggested to have important role to reduced the risk of carcinogenesis.

The antibacterial activity of T. itama and *G. thoracica* propolis in this study was categorized as weak. Previous studies showed ethanolic and hexanic extract of H.itama exhibit better activity against gram positive bacteria. Abdullah et al. (2019)(23) reported that the antibacterial activity of *H. itama* propolis is species dependent, in which they showed that the particle of *H. itama* propolis has better activity against B. subtilis, S aureus, and P. aeruginosa but not in E. coli. The flavonoid and total phenolic compounds are suggested to be responsible to antibacterial activities in previous reports (24). In this study we found that the antibacterial activity against S. aureus was not always correlated to the presence of phenolic compounds and flavonoids. This phenomenon might be caused by the presence of many other inactive compounds that found in the methanolic extract of *H. itama* and *G. thoracica* propolis. However, further study is needed to prove this theory, for instance purification of the extract using several solvents might increase the antibacterial activity of these extracts.

CONCLUSION

The methanolic extract of *H. itama* propolis contains alkaloids, terpenoids and tannins, while *G. thoracica* propolis contains flavonoids, terpenoids, saponins and tannins. Both propolis from East Kalimantan contained 875 mgGAE/100g (*H. itama*) and 880 mgGAE/100g (*G. thoracica*) phenolic compounds, respectively. *H. itama* propolis extract had better antioxidant efficacy (84% inhibition) meanwhile *G. thoracica* had better potency (IC50: 42.55 ppm). Both extracts were not effective in antibacterial activity against S. aureus.

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