p-ISSN: 1907-8056
e-ISSN: 2527-5410
AGROINTEK
Jurnal Teknologi Industri Pertanian
journal homepage: journal.trunojoyo.ac.id/agrointek

Physicochemical properties, antioxidant and antibacterial activities of honey produced by *Heterotrigona itama* and *Tetragonula laeviceps*

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Article history
Received:
10 September 2023
Revised:
20 August 2024
Accepted:
3 October 2024

Keyword
Antibacterial;
Antioxidant;
Honey;
Heterotrigona itama;
Tetragonula laeviceps

ABSTRACT

Honey is a popular healthy food that also serves as medicine. Stingless bee honey gained interest compared to regular honey due to its health benefits. This study aimed to investigate the potential health benefits of honey produced by stingless bees Heterotrigona itama and Tetragonula laeviceps, namely phenolic content, flavonoid content, antioxidant capacity, and antimicrobial activity. The research was conducted using complete randomized experimental design by two separate replications. The result shows both honeys have a pH value ranging from 3.16 to 3.43. The antioxidant capacity, total phenolic compounds, and total flavonoid compounds of both honeys were not different (p≥ 0.05). The antioxidant capacity of honey produced by Heterotrigona itama and Tetragonula laeviceps was 593 mg TE/100 and 581.58 mg TE/100 g, respectively. The total phenolic compound was in the range of 839.26-1242 mg GAE/kg, while total flavonoid was in the range 126 – 179 mg QE/ of) However, H.itama honey had the highest inhibition against Gram-positive Streptococcus mutans, while T.laeviceps had the highest inhibition against Gram-negative Pseudomonas aeruginosa. The types of local vegetation might relate to phytochemical compounds in honey. This study gave insight into another possible antibacterial compound in stingless bee honey, namely, a bioactive peptide that might act differently on microorganisms.

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DOI 10.21107/agrointek.v19i2.22290

INTRODUCTION

Honey is obtained from honeybees, and stingless bees consist mainly of glucose and fructose (Nasir et al. 2019). Honey has been known for its health benefits, namely its anti-inflammatory, anti-tumor, antibacterial, and antioxidant qualities (Bueno-Costa et al. 2016). Studies on honey produced by stingless gained interest due to its superiority compared to regular honey (Rozman et al., 2022). Stingless bee honey has more potent antimicrobial activity compared to European bee honey (Amin et al. 2018; Rao et al. 2016).

Ávila et al. (2018) reported there are 500 species of stingless bees worldwide. Syafrizal et al. (2020) reported that 12 species of stingless bee honey were identified in Indonesia. *Tetragonula laeviceps* was the most preferred by farmers, followed by *Heterotrigona itama*. *H.itama* and T. *laeviceps* belong to the family Apidae in the tribe Meliponi (Cheng et al. 2019).

Recently, research has been done to investigate the antibacterial activity of stingless bee honey. Wu et al. (2023) reported that honey obtained from *H itama* had intense antibacterial activity against gram-negative *Pseudomonas aeruginosa*. Meanwhile, Tuksitha et al. (2018) reported that honey obtained from *H. itama* had the lowest minimum inhibitory concentration (MIC) against Gram-positive *Staphylococcus intermedius* and *Staphylococcus alactolyticus*.

Phenolic compounds contained in stingless bees play an essential role as a bioactive compound (Zulhendri et al. 2022). Moreover, Leyva-Jimenez et al. (2019) reported that the antibacterial activity of stingless bee honey might be correlated with phenolic compounds.

The honey composition produced by stingless bees is significantly related to the bee species, floral source, and geography. As Syafrizal et al. (2020) reported, honey from *H. itama* colonized in Tarakan had a higher antioxidant capacity than that of colonized in Samarinda. Moreover, Kaškoniene and Venskutonis (2010) reported that collection season, storage mode, and interaction among chemical compounds and enzymes in the honey might affect the chemical composition of honey.

Therefore, this research aims were to investigate the total phenolic content, total flavonoid, and antioxidant capacity of honey produced by *H. itama* colonized in South Sumatera and *T. laeviceps* colonized East Java. Moreover, the antibacterial activity of honey produced by *H. itama* and *T. laeviceps* was also investigated.

METHODS

Stingless bee honey was obtained from PT. Beema Boga Arta (Bogor, West Java, Indonesia). All standards were obtained from Sigma-Aldrich Co. (St. Louis, USA) (gallic acid, Trolox, quercetin). The reagents of antioxidant capacity were obtained from Merck (Darmstadt, Germany): ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), potassium persulfate, phosphate buffer saline. At the same time, chemicals were obtained from Merck (Darmstadt, Germany).

Evaluation

Measurement of pH value

The pH meter (Eutech pH 700 Benchtop pH Meter, Sternberk, Czech) was calibrated using pH solutions of 4, 7, and 10. Honey was diluted 4 times, and then the pH was measured using a pHmeter

Total Phenolic compound

Total phenolic compounds were determined using the Folin-Ciocalteau method (Dewanto et al., 2002). A honey sample of 0.1 ml was mixed with 0.1 ml of Folin-Ciocalteau reagent. The mixture was set for 6 minutes at room temperature to allow reaction, then 1 ml NaCO3 7% and 1 ml of distilled water was added, and the mixture was held for 90 min incubation. The absorbance was measured at 760 nm using a Mapada UV-1 100 spectrophotometer (Mapada UV-1 100, Shanghai Mapada Instrument Co., Ltd., Shanghai, China) and expressed as mg GAE/ 100 g sample.

Total flavonoid content (TFC)

Total flavonoid content was determined based on a method proposed by Sulastri et al. (2018). The sample of 1 ml was mixed with 3 mL ethanol 96%. The mixture was then added with 0.2 ml 10% $AlCl_3$ followed by 0.2 ml of 1 M CH_3COOK and 5.6 distilled water. The absorbance was measured at 376 nm using a UV-100 Mapada (Mapada UV-1 100, Shanghai Mapada Instrument Co., Ltd., Shanghai, China) spectrophotometer. The result was expressed as mg quercetin, equivalent to 100 grams of honey.

Analysis of Antioxidant Capacity

The antioxidant capacity was measured based on the Electron transfer mechanism by TEAC assay as proposed by Re et al. (1999). The $ABTS^{\bullet+}$ solution was made by adding 5 ml of 7 mM ABTS to 88 µl of 140 mM $K_2S_2O_8$ in the dark and held for 24 h at room temperature before use. The $ABTS^{\bullet+}$ stock solution was diluted in phosphate buffer saline (PBS). to get an absorbance of 0.700 ± 0.020 at 734 nm. Antioxidant capacity was measured by mixing 5 µl of diluted sample with 1 ml of $ABTS^{\bullet+}$ radical solution. The absorbance at 734 nm was recorded using a UV-100 Mapada spectrophotometer by analyzing the decolorization of the $ABTS^{\bullet+}$ at 734 nm after 4 min at 30°C using PBS as a control. The standard curve was made using Trolox at 0.5- 5 mM.

Antimicrobial activities using the Agar well diffusion method

The antimicrobial activity of honey obtained from *H. itama* and *T. laeviceps* was determined by the agar diffusion method following the method proposed by Boorn et al. (2010). Five bacteria were used: *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimorium*, and *Streptococcus mutant*. Nutrient agar was spread with 1 ml of test microbe culture. Each plate contained three wells, and 1 ml of honey solution was filled in the well. Samples were incubated at 37°C for three days, and the inhibition zone was measured on day three.

Statistical analysis

Data was obtained from two separate replications followed by an Analysis of variance using Microsoft Excel.

RESULTS AND DISCUSSION

Polyphenolic compound and Antioxidant activity of Honey

Both honeys had pH values ranging from 3.16 to 3.43. This result was in agreement with Chuttong et al. (2016). Figure 1 shows the antioxidant capacity, total flavonoid content, and total phenolic content of honey produced by *H. itama* and *T. laeviceps* were not different (P≥0.05). Mahani et al. (2022) reported that the total flavonoid content and antioxidant capacity of honey obtained from *H. itama* was higher than that of *T. laeviceps.*

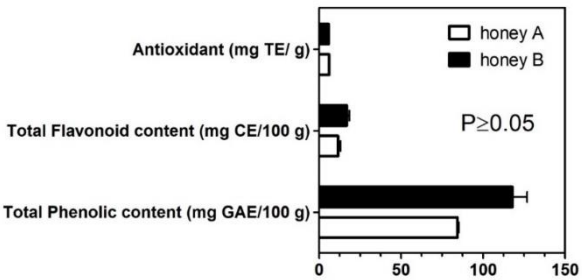


Figure 1 Antioxidant capacity, total flavonoid content, and total phenolic content of honey produced by (a) *H. itama* and (b) *T. laeviceps*

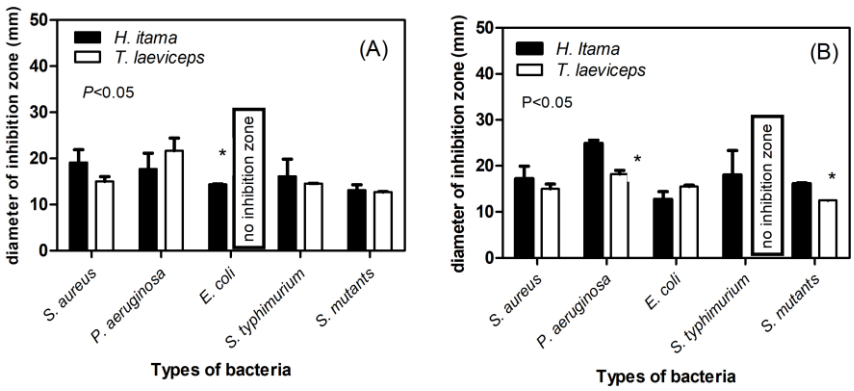


Figure 2 The inhibition zone of (A) 100% honey and (B) 50% honey solution determined by Agar well diffusion. Bars represent the standard deviation

Table 1 Minimum inhibition concentration (mic) of honey produced by H. itama and T. laeviceps against bacteria

Bacteria	MIC value of honey produced by stingless bee (% v/v)	
	H. itama	*T. laeviceps*
Staphylococcus aureus	50	50
Pseudomonas aeruginosa	50	12.5
Escherichia coli	25	100
Salmonella typhimorium	50	100
Streptococcus mutant	12.5	50

The antioxidant capacity of both honeys were in the range of 5.81 – 5.93 mg TE/g. This result was in the range of antioxidant capacity proposed by Attanzio et al. (2016). Both honey obtained from *H. itama* and *T. laeviceps* has a total phenolic content and total flavonoid content that is higher than Cheng et al. (2019) but in the range of TPC and TFC reported by Attanzio et al. (2016).

Although honey is obtained from different bee species and different places of bee colonization, honey composition is related to vegetation as a nectar source for the bees. Mahani et al. (2022) reported there were 29 plants found in East Java that use as a nectar source for *T. laeviceps,* namely durian, cassava, coconut, mango, banana, passion fruit, pineapple, jackfruit, rubber tree, jatropha, starfruit, sunflower, cacao, coffee, guava, litchi, Siamese pumpkin, bay leaves, sengon wood, stink bean, mimosa, calliandra, dandelion. Meanwhile, Priawandiputra et al. (2020) also reported these 29 plants were available in South Sumatera and used as nectar sources for *H. itama*. This might explain the similar value (P≥ 0.05) of antioxidant capacity, total phenolic content, and total flavonoid content of both honeys.

Antimicrobial Activity of Honey

Figure 2 shows the antibacterial activity against five bacteria, namely *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimorium*, and *Streptococcus mutant*. Both kinds of honey produced by *H. itama* and *T. laeviceps* had antibacterial activity. However, after 50% dilution, honey produced by *T. laeviceps* showed no inhibition zone against *Salmonella typhimorium*. Interestingly, at 50% dilution, honey produced by *H. itama* showed a higher inhibition zone ($P < 0.05$) against *P. aeruginosa* and *S. mutant* than that of *T. laevicaeps*.

Honey produced by *H. itama* and *T. laeviceps* showed different MIC values against several bacteria (Table 1). Interestingly, honey produced by *H. itama* had antibacterial activity against *S. mutant* at a low MIC value of 12.5% (Figure 3). Meanwhile, honey produced by *T. laeviceps* had antibacterial activity against *P. aeruginosa* at a low MIC value of 12.5% (Figure 3 Inhibition zone of honey obtained from T. laeviceps against Gram-negative Pseudomonas aeroginosa at different concentrations

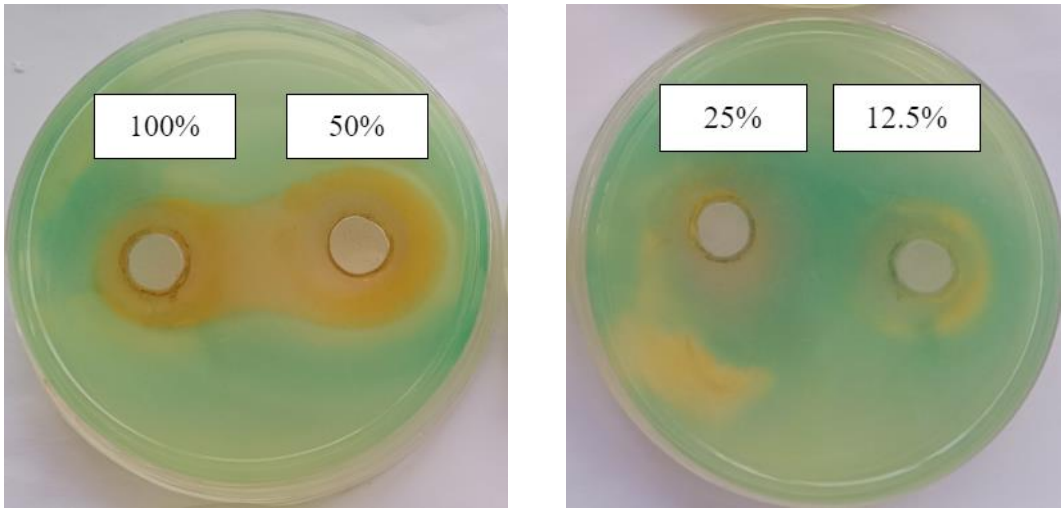
). However, both honey produced by *H. itama* and *T. laeviceps* have antibacterial activity against *E. coli* at 100% and 50% (Figure 4)

Both stingless bee honey have antibacterial activity against gram-positive and gram-negative bacteria. *S. mutant* was the most sensitive bacterial strain against *H. itama* honey, while *S. aureus, S. tyhpymorium,* and *P. aeruginosa* were the least sensitive. *P. aeruginosa* was the most sensitive bacterial strain against *T. laeviceps,* while *E. coli* and S. *typhimorium* were the least sensitive. This result was in agreement with Hasali et al. (2018). Honey obtained from both *H. itama* and *T. laeviceps* has the least antibacterial activity against gram-negative bacteria. Interestingly, honey obtained from *T. laeviceps* had intense antibacterial activity against gram-negative *P. aeruginosa,* indicated by a MIC value of 12.5% (v/v). Diluting honey might increase antibacterial activity due to activation of H_2O_2 by glucose oxidase during dilution (Deglovic et al. 2022)

Wu et al. (2023) reported each phenolic compound was significantly correlated with antibacterial activities. It is noteworthy that although the total phenolic content, total flavonoids, and antioxidant capacity from honey produced by *H. itama* and *T. laeviceps* were not different, both kinds of honey showed antibacterial activities against different bacteria strains. Ávila et al. (2018) reported that HMF was

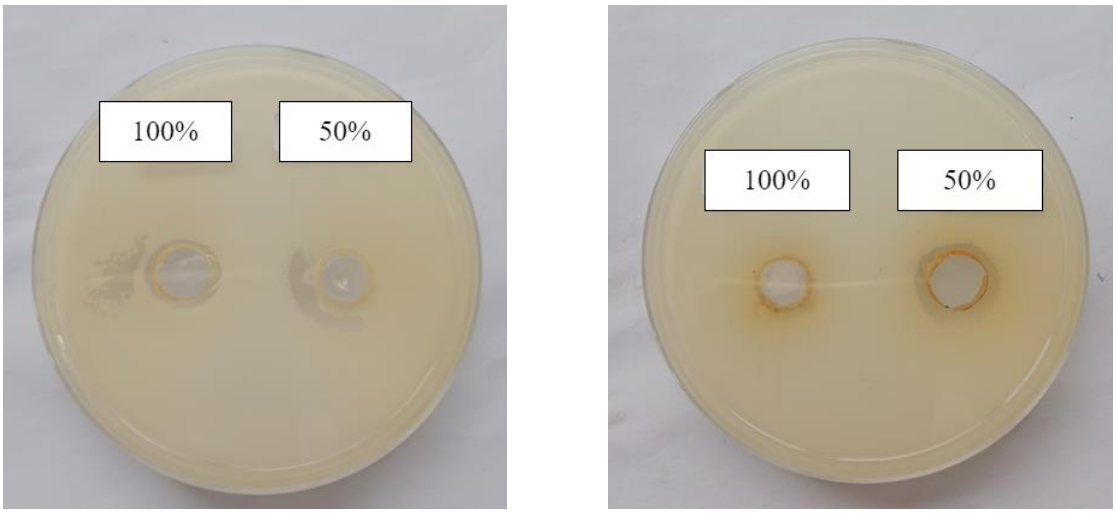
found in honey produced by *T. laeviceps* while it was not detected in honey produced by *H. itama*. Although Nishio et al. (2016) proposed hydroxymethyl furfural (HMF) as an antimicrobial, Nafea et al. (2011) reported that HMF did not show antibacterial activity against *P. aeruginosa*.

Moreover, Jantakee and Tragoolpua (2015) reported that bioactive peptides contained in honey might also contribute to its antibacterial activity. Sahlan et al. (2019) reported that glucose dehydrogenase (GDH) was detected in honey produced by *T. laeviceps,* but it was not detected in honey produced by *H. itama*. This might explain the different antibacterial activity of honey obtained from *H. itama* and *T. laeviceps,* as also explained by Deglovic et al. (2022). GDH was used as a supplement to glucose oxidase to produce H_2O_2 as an antimicrobial through oxidation (Erban et al., 2019). This might explain the antibacterial activity of honey produced by *H. itama* and *T. laeviceps* against bacterial strains.



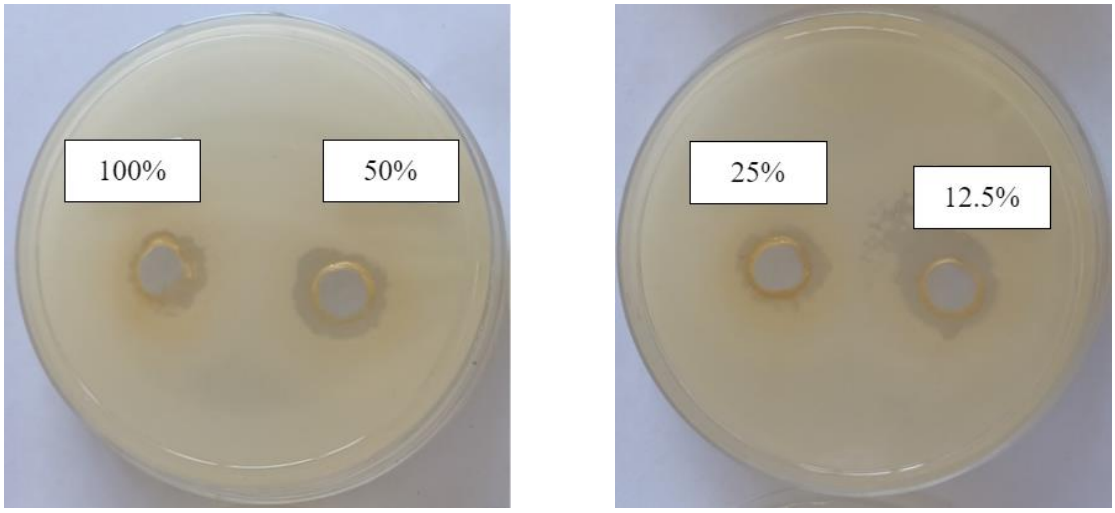
A B

Figure 3 Inhibition zone of honey obtained from T. laeviceps against Gram-negative Pseudomonas aeroginosa at different concentrations



A B

Figure 4 The inhibition zone of honey was obtained from (A) H. itama and (B) T. laevicaeps against E. coli at 100% and 50% (v/v) concentrations



A B

Figure 5 The inhibition zone of honey was obtained from *H. itama* against Gram-positive *Streptococcus mutants* at different concentrations

CONCLUSION

The study shows that the total phenolic content, total flavonoid content, and antioxidant capacity from different species in the exact geographical location were not different since the vegetation as a nectar source was found in both locations. However, the differences in the composition of phenolic and flavonoid from each stingless bee honey vary in antibacterial activity against bacterial strains. Further research is needed to investigate bioactive compounds in honey other than phenolic and flavonoids that have antibacterial activity.

ACKNOWLEDGEMENT

Financial support for student fellowship to Zulfikar Fadil and Trisya Nur Adzni Destianti was provided by the Ministry of Education under the Magang Studi Independent Bersertifikat (MSIB) program. We are grateful to Beema Honey for providing stingless bee honey.

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