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Quality Analysis, Phenolic and Flavonoid Content, and Antimicrobial Activity of Stingless Bees Honey (*Heterotrigona itama*)

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ABSTRACT

Stingless bee (*Heterotrigona itama*) honey has been utilized as a therapeutic ingredient for treating infectious illnesses. This study aims to investigate the quality of stingless honey, total phenolic and flavonoid content, and antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Honey quality parameters evaluated included organoleptic tests, diastase enzyme activity values, hydroxymethylfurfural (HMF) content, water content, reducing sugar content, honey acidity, and lead (Pb) contamination with test procedures based on the Indonesian National Standard (SNI) 8664: 2018 on honey. The antimicrobial activity test uses the well diffusion method at various concentrations, i.e., 5, 10, 15, 20, and 30%. The results showed four honey quality parameters, i.e., organoleptic, HMF content (0.0054 mg/kg), water content (27.41%), and lead metal contamination levels (0.001198 mg/kg). Honey contained total phenolic content of 6.56 ± 0.03 mg GAE/g and total flavonoid levels of 5.80 ± 0.03 mg QE/g. The stingless honey could stop the growth of *Staphylococcus aureus* and *Escherichia coli* at the lowest concentration of 5%, with inhibition zone diameters of 14.7 and 9.3 mm diameters. Stingless bee honey did not affect *Candida albicans* growth.

Keywords: Antimicrobial activity, *Heterotrigona itama*, honey quality, total flavonoid content, total phenolic content

Introduction

Honey is a naturally sweet-tasting liquid produced by honey bees from floral essences and other plant ingredients. Honey bees convert plant nectar into honey, stored in beehive cells (Hakim et al., 2021). Honey has always been utilized as a food and medical source (Huda, 2013). Traditional use of honey to treat infectious disorders such as upper respiratory and digestive tract infections has demonstrated this. Honey contains a hydrogen peroxide compound that is an antiseptic and can treat infected wounds (Dewi et al.,2017). In addition, the antiseptic properties of honey are due to the presence of phenolic compounds. Phenol has antibacterial activity and can inhibit the activity of β-lactamase enzymes generated by Gram-negative bacteria and prevent the formation of biofilms (Mandal et al., 2017).

The *Heterotrigona itama* bee, also known as *kelulut*, is stingless. Cultivating stingless bees offers various benefits over other honey bee cultivations, including simple maintenance, simple colony development, and no famine (Karnia et al., 2019). Honey from stingless bees has a distinct scent, sour flavor, and clearer color. Complex enzymatic components, including invertase, diastase, catalase, glucose oxidase, phosphatase, and protease, are present in this form of honey (Fatma et al., 2017). Moreover, this honey contains non-enzymatic components like proteins, carotenoids, amino acids, flavonoids, and phenolic acids (Emmasitah et al., 2020; Hakim et al., 2021).

Honey quality parameters can be used to describe the freshness and authenticity of honey. Honey that meets the quality requirements of honey is honey of good quality. Honey quality is proportional to honey content. The Indonesian National Standard (abbreviated SNI) 8664-2018 regulates honey quality in Indonesia.

According to the previous definition, honey is regarded as a supplemental treatment for a variety of ailments, notably infectious ones. However, nothing is known regarding the quality of kelulut honey or its antimicrobial properties. This study looked at water content, acidity, reducing sugar content, hydroxymethylfurfural (HMF) content, diastase enzyme levels, and metal lead levels (Pb).

Research Methods

Instruments and Materials

LAFC (Laminar Air Flow Cabinet), autoclave, incubator, Refractometer, and vortex.

Samples of stingless bee honey were harvested in March 2022 from Barabung Village, Aceh Besar District. Bacteria *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Candida albicans* ATCC 10231, Mueller Hinton Agar (MHA), Sabouraud Dextrose Agar (SDA), Amoxicillin, Gentamicin, Fluconazole, Folin Ciocalteu reagent, quercetin, and gallic acid.

Experiments

1. *Analysis of honey*
   Water content

The water content of stingless bee honey was tested using the AOAC Official Method 969.38-1999. Water content is determined using a refractometer. The water content is determined by comparing the refractive index value and water when the temperature reaches 20°C (Ridoni et al., 2020).

Acidity test

The acidity of honey was tested according to the Codex Standards for Sugars (including honey); 10 gs of honey was put in an Erlenmeyer flask. Then 75 mL of distilled water was added, followed by 4-5 drops of phenolphthalein indicator solution. The solution was adjusted with 0.1N NaOH until the end point remained stable for up to 10 seconds. The amount of 0.1N NaOH used in the titration was measured. As an alternative, the sample is titrated, and the pH is measured to pH 8.3 using a pH meter. (Ridoni et al., 2020; Wulandari et al., 2017).

Reducing sugar content

For covering the blue spot on the refractometer, a few drops of honey are dripped on the plate. The acquired data are listed on a scale of total sugar content values. Brix units are used to express value. Degree Brix, commonly referred to as % Brix, is a unit of measurement for the level of sugar in liquids (Karnia et al., 2019).

Hydroxymethylfurfural (HMF) content

HMF levels are calculated referring to SNI 8664 regarding honey. Blank solutions were prepared employing Carrez I and Carrez II solutions, each was given 0.5 mL, then 50 mL of distilled water was added. Sample preparation was carried out to clarify the solution by precipitating the protein. Five gs of honey dissolved in distilled water up to 25 mL. 0.5 mL of Carres I and Carrez II solutions were added to the sample solution. The solution was then homogenized with 50 mL of distilled water. The sample solution was filtered until it was crystal clear. 1 mL of the filtered solution was mixed with 19 mL of distilled water. 10 mL of 0.2% NaHSO3 was mixed with 9 mL of 0.1% $NaHSO_3$ to make the reference solution. A UV-Vis spectrophotometer was used to measure the absorbance of the sample solution and solution comparison at 284 nm and 336 nm, respectively. (Koesprimadisari et al., 2016)

Diastase Enzyme content

Starch and distilled water were both boiled for 15 minutes at 40°C. Then, pipette up to 10 mL of distilled water and 5 mL of starch into a 25 mL Erlenmeyer and stir until homogeneous. The mixture was heated for 15 minutes at 40°C. A 25 mL test tube containing 10 mL of iodine solution was then filled with 1 mL of distilled water and starch, which was then homogenized. A wavelength of 660 nm was used to measure the solution. If the absorbance is still below 0.76 ± 0.02, the procedure is repeated, and measurements are taken while adding distilled water until the absorbance is obtained (Akuba & Pakaya, 2020).

In a beaker glass, five gs of the solution were added. afterwards, 2.5 mL of pH 5.3 acetate buffer solution was included and thoroughly mixed. A 25 mL volumetric flask carrying 1.5 mL of sodium chloride solution was used to transfer the solution. The mark was then filled with distilled water in a 50 mL Erlenmeyer flask and pipette 10 mL of the sample solution. Then heat for 15 minutes in a water bath at 40°C with a volumetric flask containing a standard starch solution. 5.0 mL of starch solution was pipetted into the sample solution after 15 minutes. The sample solution was then heated once more in the water bath for 15 minutes at 40°C. Once more, 1 mL of the sample solution was pipetted into a test tube containing 10.0 mL of 0.0007 N iodine. After that, distilled water was added and homogenized in the appropriate volume to the standardized starch. The absorbance was calculated at 660 nm using distilled water as a blank. Then, until an absorption of less than 0.235 was attained, the solution was sampled at intervals of 15 minutes. The method for calculating the levels of the diastase enzyme was used to examine the findings of the honey diastase enzyme activity test (Akuba & Pakaya, 2020).

Lead metal (Pb) content

Lead metal levels were tested using an atomic absorption spectrophotometer (AAS). Using a wet digester, honey samples were made utilizing the wet destruction process. 1.5 gs of honey, 1.5 mL of HNO3 acid, and 1.5 mL of H_2O_2 acid were mixed together and set aside for three hours. The solution was then digested for three hours at 550°C in a wet digester. The sample should then be removed from the wet digester, added to HNO_3, and filtered through Whatman 40 filter paper. Then put into a 100 mL measuring flask, add 1000 µg/mL of lead mother liquor as much as 10 µL, dilute with 5% HNO_3 to mark, and then homogenized. The sample's absorption was analyzed using an atomic absorption spectrophotometer (AAS) at λ 283.3. The lead concentration in the honey sample was calculated using a standard lead solution calibration curve. The lead level in honey is calculated after correcting the dilution factor (Saputra et al., 2018).

2. *Phytochemical test*

Alkaloid

A sample of 1 g of honey was weighed and put into a test tube, then added Dragendroff reagent. The same treatment was repeated

using Meyer's and Wagner's reagents. The production of a precipitate indicates the presence of alkaloids. (Khairunnisa et al., 2020)

Flavonoids

One g of honey is weighed and placed in a test tube as a sample. After that, it was boiled and protracted in 4 mL of ethanol. Next, 3 mg of Mg metal was added, along with some concentrated HCl. Flavonoids are present if a reddish-orange to purple-red color develops. If an orange-yellow color appears, flavones, chalcones, and aurons are present (Khairunnisa et al., 2020).

Phenolic compunds

One g of honey must be added to a test tube with about 1% $FeCl_3$ reagent. Colors such as red, green, purple, blue, and black indicate the present phenolic compounds (Khairunnisa et al., 2020).

Tannins

Four mL of water and one g of honey should be added to a test tube. It is heated for a further 1-2 minutes. A reagent solution containing 1% FeCl3 was filled to the filtrate. The emergence of a black-green or dark blue color indicates a successful tannin reaction (Khairunnisa et al., 2020).

Saponins

Add distilled water and two gs of honey in a test tube until the sample is completely covered. After a 2- to 3-minute boil, let it cool. Stable foam production indicates successful saponin outcomes (Ngajow et al., 2013).

Steroids and triterpenoids

In a test tube, place two gs of honey. Add anhydrous acetic acid until the sample is completely covered. Let stand for 15 minutes. Add a few sprays of strong sulfuric acid to six drops of the solution. Blue indicates the presence of steroids, while red, orange, or purple indicates triterpenoids (Ngajow et al., 2013).

3. *Total phenolic content determination*

To get a 1000 ppm concentration, 10 mg of honey was dissolved in 10 mL of ethanol. Then, 0.3 mL of the solution was mixed with 1.5 mL of Folin-Ciocalteu reagent. After the solution had stood for 3 minutes, 1.2 mL of 7.5% sodium bicarbonate solution was added. The solution was incubated for 90 minutes at room temperature. The absorbance was measured using a UV-Vis spectrophotometer at a maximum wavelength of 765 nm. Total phenolic was calculated using the gallic acid standard curve's linear regression equation $y = ax + b$ with R^2= 0,999. (Ramadhani et al., 2020) Gallic acid equivalent per g of material (mg GAE/g) is used to quantify phenol levels.

4. *Total flavonoid content determination*

10 mg of honey dissolved in ethanol to 10 mL of ethanol to achieve a concentration of 1000 ppm. Then, 1 mL of the solution was combined with 8 mL of 5% acetic acid and 1 mL of 10% $AlCl_3$. For 30 minutes, the solution was incubated. A UV-Vis Spectrophotometer was utilized to detect absorption at a wavelength of 436 nm. Total flavonoids were estimated using the conventional quercetin curve's linear regression equation $y = ax + b$ with R^2= 0,999 (Ramadhani et al., 2020). In units of mg quercetin equivalent/*g sample (mg QE/g), flavonoid levels are indicated.*

5. *Antimicrobial activity test*

Antimicrobial activity test against *Staphylococcus aureus* and *Escherichia coli* was conducted on MHA media, and *Candida albicans* was performed on SDA media employing the well method. Each microbial suspension was prepared with turbidity equivalent to 0.5 Mc Farland. Holes (6 mm) were made, with as many as seven holes in each medium. Stingless bee honey was made in various concentrations (w/v) 5; 10; 15; 20, and 30%. A 30 µL sample solution was taken and dripped into the well. Amoxicillin 0.05% was used as a positive control against *S. aureus*, 4% gentamicin was used as a positive control for *E. coli*, and 1% fluconazole was used as a positive control for *C. albicans*. Aquadest was used as a negative control. Observations were made after 24 hours of the bacterial incubation period and 48 hours of the fungal incubation period. A caliper was used for measuring the inhibitory area diameter in millimeters (mm).

Data Analysis

The datasets gathered were analyzed employing the descriptive analysis method.

Result and Discussion

Honey Quality Analysis

A criterion that can be used to measure the viability of honey for consumption is its quality. Both internal and external factors affect the honey's quality. The health of honey bee colonies, including their capacity to produce honey, overall population size, and the number of broods, might be considered internal factors. These three factors will determine the situation of the honey bee colony in the hive. A robust bee colony will be more effective at controlling the hive's temperature, and the honey-evaporation process will be flawless, allowing the honey to ripen more rapidly. Weather and climate conditions, air humidity, the type of plant used to feed honeybees, the age of harvest, the maturity level of the honey, the treatment, the harvest period, and post-harvest measures are examples of external factors (Fatma et al., 2017).

Table 1. Results of honey quality analysis

Test Type	Unit	Test Results	SNI 8664:2018	Information
Organoleptic test				
Smell		Normal	Taste of honey	According to SNI
Flavor		Normal	Taste of honey	According to SNI
Laboratory test				
Diastase enzyme activity	DN	0.58	Min 1	Not appropriate
Hydroxymethylfurfural (HMF)	mg/Kg	0.0054	Max 40	According to SNI
Water content	% b/b	27,41	Max 27.5	According to SNI
Reducing sugar (glucose)	% b/b	28,25	Min 55	Not appropriate
Acidity	mL NaOH/Kg	293.3	Max 200	Not appropriate
Pb contamination	mg/Kg	0.001198	Max1.0	According to SNI

Tabel 2. Phytochemical test results

Metabolite groups	Results
Alkaloid	Negative
Flavonoid	Positive
Phenolic	Positive
Tannin	Positive
Saponin	Positive
Steroid	Negative
Triterpenoid	Positive

The diastase enzyme is a natural compound from bee saliva and is added to the honeycomb during the honey ripening process (Jannetta, 2022; Lastriyanto & Cahyani, 2021). The diastase enzyme can convert complex carbohydrates into simple carbohydrates, namely glucose, and fructose. Hydroxymethylfurfural (HMF) is a cyclic aldehyde derived the value of the diastase enzyme obtained was 0.58 DN. This value does not meet SNI standards. from sucrose, fructose, and other monosaccharides with six C atoms. In an acidic environment, this decomposition process takes place more quickly with the help of heat.

HMF levels indicate the quality of honey, characterized by excessive heating or the addition of inverted sugar to honey (Ariandi & Khaerati, 2017; Koesprimadisari et al., 2016). HMF levels were determined at 0.0054 mg/kg. This value meets the SNI 8664 requirement for stingless bee honey. The amount of water in honey is a key indicator of its purity and freshness. As the water content of honey decreases, its quality rises.

Good honey has a water content of between 17% and 21% (Ariandi & Khaerati, 2017). At 27.41%, the water content value was measured. This value complies with SNI 8664 requirements. One of the hallmarks of stingless bee honey is its high-water content (Hakim et al., 2021). Honey quality analysis can be seen in Table 1.

The monosaccharide class of carbs, specifically glucose and fructose, make up the majority of honey's ingredients. Glucose can reduce electron acceptor compounds, called reducing sugars (Ridoni et al., 2020). Reducing sugar levels in honey are calculated as glucose levels. The reduced sugar content was obtained at 28.25%. This value does not meet the SNI 8664 standard. The honey acidity test used the acid-base titration method referring to SNI 8664.

Honey's acidity reveals how much free acid there is in each kilog. The acidity value of honey was obtained at 293.3 mL of NaOH/Kg. This value does not meet the SNI 8664 standard. The honey of stingless bees is reported to be more acidic than that of other bee species. The presence of organic acids, including gluconic acid, pyruvic acid, malic acid, and citric acid, as well as inorganic ions like phosphate, sulfate, and chloride, determines how acidic honey is (Fatma et al., 2017). The amount of water in honey affects its acidity as well. Since stingless bee honey has been shown to contain more water than other types of honey, it has a watery texture. Because it causes fermentation, honey with a relatively high-water content has a lower acidity (Karnia et al., 2019). One of honey's many qualities is that it can dissolve metals or be corrosive. As a result, honey processing impacts the metal content of honey. Insecticide use on plants, gas emissions from motor vehicles, lead-polluted water, and metal tools are some sources of lead metal contamination in honey. Lead is not naturally occurring; it is typically found in mineral ore with other metals like zinc, silver, and copper. The sample of stingless honey contained 0.001198 mg/kg of lead metal contamination. This value complies with SNI 8664 requirements.

Phytochemical Test

Using color reagents, the phytochemical test procedure was carried out qualitatively. Table 2 displays the findings of the phytochemical test on stingless honey.

Flavonoids, tannins, saponin, steroids, and phenolic secondary metabolites are all in stingless bee honey. To support the author's theory, tests were done to see the concentration of secondary metabolites in samples of Stingless bee honey bees.

Total Phenolic and Flavonoid Content

Using a UV-Vis spectrophotometer, samples of stingless bee honey were tested for their total phenolic content using the Folin-Ciocalteau method. This method's basic idea is based on how well standard gallic acid reduces the hydroxy phenol groups. Gallic acid is a hydroxybenzoic acid derivative classified as a simple phenolic acid.

Table 3. Results of the antimicrobial activity test of stingless bee honey

Honey concentration (% w/v)	Inhibition zone diameter (mm)		
	S. aureus	*E. coli*	*C. albicans*
5	14,7±0.15	9,3±0.1	0
10	22.5±0.15	14,6±0.05	0
15	26,7±0.25	18.5±0.1	0
20	31,4±0.2	22,1±0.05	0
30	35,8±0.6	25±0.4	0
Positive control	47,2±0.7	37±0.4	38.5±0.15
Negative control	0	0	0

Gallic acid has a relatively high reactivity to the Folin-Ciocalteu reagent compared to other phenolic compounds. It is relatively stable and pure, so it is used as a standard intesting total phenolic content (Rahmi et al., 2021). Gallic acid equivalent (GAE)/g of sample was used to express the amount of phenol in the sample. The test results for the total phenolic content of the stingless bee honey sample were 6.56 ± 0.03 mg GAE/g. Stingless bee honey from Pandeglang has a total phenolic content of 18.50 mg GAE/g (Abdillah et al., 2022). Differences in phenolic content can be influenced by the type of nectar that bees feed on, and vary according to the region where the honey is produced (Becerril-sánchez et al., 2021)

The total flavonoid content test was carried out using the colorimetric method, adding 10% $AlCl_3$ reagent, 5% acetic acid, and quercetin as standard. The principle of the $AlCl_3$ method is the formation of stable complex compounds with ketone groups at C-4 and hydroxyl groups at C-3 or C-5 from flavones or flavanols that produce a yellow solution (Sari & Ayuchecaria, 2017). Quercetin was selected for a standard solution considering it is one of the flavonoid compounds which shall react with $AlCl_3$ to form complex compounds (Haeria et al., 2016). Quercetin equivalent (QE) measurements of the flavonoid levels were represented as mg/g of sample. The stingless bee honey sample tested positive for total flavonoid levels at 5.80±0.03 mg QE/g. Stingless bee honey from Pandeglang has a total flavonoid content of 10.80 mg QE/g (Abdillah et al., 2022). Likewise, differences in flavonoid content can be influenced by the type of nectar that bees feed on, and vary according to the region where the honey is produced (Becerril-sánchez et al., 2021)

Antimicrobial Activity Test of Stingless Bee Honey

Test results of Stingless bee the activity of stingless bee honey against *S. aureus* bacteria started with the smallest concentration of 5%, producing an inhibition zone of 14.7 ± 0.15 mm, and the largest concentration of 30%, honey activity against bacteria and fungi can be seen in Table 3.

The activity of stingless bee honey against *S. aureus* bacteria started with the smallest concentration of 5%, producing an inhibition zone of 14.7 ± 0.15 mm, and the largest concentration of 30%, producing an inhibition zone of 35.8 ± 0.6 mm. The activity of stingless bee honey against *E. coli* bacteria at the smallest concentration of 5% produced an inhibition zone of 9.3 ± 0.1 mm and the largest concentration was 30% with an inhibition zone of 25±0.4 mm. In Figure 1, the inhibition zone is depicted. The antibacterial activity of stingless bee honey increases with increasing variations in honey concentration. This research is supported by Nur et al., (2019), who reported that stingless bee honey has significant antibacterial activity towards *S. aureus* and *E. coli* bacteria. The activity test results for the two bacteria can prove that stingless bee honey bee samples may stop the development of both Gram-positive and Gram-negative bacteria. Stingless bee honey from Barabung Village, Aceh Besar, has a larger inhibition zone diameter against *S. aureus* bacteria than *E. coli* at each concentration variation.

Activity test of stingless bee honey against *C. albicans* did not produce an inhibition zone. The activity test results of stingless bee honey against *C. albicans* fungus do not follow the literature, which states that the content of secondary metabolites such as flavonoids, phenolics, tannins, saponins, and triterpenoids can inhibit the growth of the fungus. This is presumably because the concentration of stingless bee honey used is low, so the secondary metabolites in honey have not been able to damage the defense of fungal cells. The fungus *C. albicans* has a cell membrane composed of proteins and lipids; This allows for complex bonds between antimicrobials and ergosterol in fungal cells so that cell organelles such as nucleic acids will emerge.

Phenolic compounds have antimicrobial potential due to the mechanism of protein binding, which can harm cell wall biosynthesis and cell membranes. Flavonoids Inhibit microbial growth by interfering with cell membrane function and reducing cell metabolism in the production of ATP and macromolecular biosynthesis. Tannins are lipophilic polyphenol derivatives that can interact with lipids and amino acids, causing membrane and cell wall permeability damage.

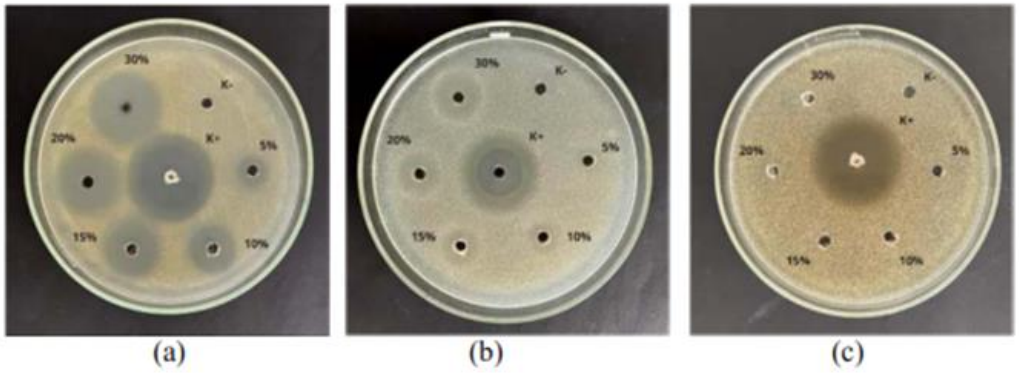

Figure 1. Activity test results of honey (a) *S. aureus* (b) *E. coli* (c) *C.albicans*

Triterpenoids inhibit the growth of antimicrobials by interfering with the permeability of the cell wall and disrupting the integrity of the membrane of bacterial cells (Surbayanti et al., 2022). Furthermore, tannins can interact with polysaccharides to form complex compounds that can damage the bacterial cell wall. Saponins have a significant function. The development of complexes between saponins and extracellular proteins can affect the permeability of the bacterial cell membrane (Bamasri, 2021).

Conclusion

Stingless bee honey originating from Barabung Village, Aceh Besar, meets the criteria for organoleptic parameters, smell, flavor, HMF, water content values, and lead Pb contamination. However, the quality of SNI is does not met the parameters. Stingless bee honey has a total phenolic content of 6.56 ± 0.03 mg GAE/g and a total flavonoid content of 5.80 ± 0.03 mg QE/g. Stingless bee honey has significant antibacterial activity against *S. aureus* and *E. coli* bacteria but does not have antifungal activity against *C. albicans*.

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