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Physicochemical and Microbiological Analysis of Stingless Bees Honey Collected from Local Market in Malaysia

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Abstract: *The growing demand for honey in the market has led to the occurrence of the tampering honey with foreign substances and increases the production of artificial honey. Due to this concern, this study works on the physicochemical and microbial characterization of stingless bee honey. The physicochemical analysis showed that the honey possessed pH (2.51–3.26), free acidity (121.1 to 318.7 meq/kg), moisture (19.4–30.9%), electrical conductivity (0.33–0.69 mS/cm), ash content (2.75–4.31 g/100g), Hydroxymethylfurfural (HMF) content (35.4 to 461.7 mg/kg) and diastase activity (2.71 to 6.11 DN). Also, sugar profile of honey showed that the honey contained fructose (15.03–32.52 g/100g), glucose (12.17–34.55 g/100g) and sucrose (0.01–7.29 g/100g). The harvested honey, H1, and H2 have the highest potential to become an antibacterial agent to treat disease compared to commercial honey samples because they were active against gram-negative bacteria. All analyzed samples were within the maximum limit of the quality criteria set by the Malaysian Kelulut Standard and Codex Alimentarius except for free acidity, HMF, and Diastase Number. All the data obtained is vital in order to create a specific statute for stingless bees honey in Malaysia that may help to protect the consumer from purchasing adulterated honey.*

Keywords: *honey; adulteration; physicochemical; microbiological; quality*

■ INTRODUCTION

Stingless bees are small, all black creatures that commonly reside in the tropical and subtropical regions of the world, such as Southeast Asia and tropical America. Stingless bees are very special insects as they can produce three different products; honey, propolis, and bee bread. In addition, stingless bees are also valued as an effective pollinator for both wild and cultivated crops [1]. Honey is a natural sweetener that is widely used for various applications where it contains approximately 200 distinct chemical compositions including 80–85% of carbohydrates such as fructose and glucose [2]. Besides that, water, proteins, and amino acids, ash, hint of enzymes, vitamins and phenolic compounds also constitute almost 15–20% in honey [2]. Nevertheless, the contents of honey differ depending on the types of plants as well as the nectar which the bee consumes [2].

The authenticity of honey is specified internationally by the Codex Alimentarius and European Legislation while locally; stingless bee honey is defined by Malaysian Standard Kelulut (Stingless Bee). The characterizations of honey, especially by physicochemical analyses, are well described by both standards [3]. Moisture content is the most popular criterion for stingless bees honey followed by free acidity, sugar profile, pH, HMF, ash content and electrical conductivity [4]. The moisture content was important as it influenced many other parameters in honey such as sugar content, hydroxymethylfurfural (HMF) and microbial properties. Based on the past studies, stingless bee honey has been reported to have a higher moisture content, higher electrical conductivity, lower enzyme activity, higher free acidity, and lower glucose and fructose content compared to *Apis mellifera* honey [4,32].

Microorganisms may influence the quality or safety of honey. Yeast and spore-forming bacteria are mostly found in honey, but to our concern, there are no bacteria causing disease had been identified in honey [5]. Microbial contaminations commonly occur in honey are from primary sources where it is difficult to control. The sources include pollen and nectar sources, the digestive system of honey bees, dirt, air and soil [6]. Good manufacturing practices may be the practical way to control the sources of contagion that are mostly found in secondary sources particularly from honey post-harvest including air, food operators, cross-contamination, equipment, and buildings [6-7].

Honey adulteration refers to the immoral act of producers by adding sugar syrups into natural product [8]. Commonly, adulterants such as water, sucrose, inverted sugar, hydroxymethyl cellulose, dextrin, and starch have been detected by routine analysis of physicochemical [9-10]. Adulteration activity of honey has increased the awareness among the honey consumers about the quality and purity of the commercial honey in the market. Thus, the study aimed to assess the quality of harvested honey and commercial stingless bee honey available in Malaysia market concerning the physicochemical properties and microbial profile.

■ EXPERIMENTAL SECTION

Materials

In this study, six samples of stingless bees honey were collected from different regions in Malaysia (Table 1). Two samples, H1 and H2, were harvested from Universiti Malaysia Pahang stingless bee farm and Aqif Kelulut Farm, Pekan. Four samples of commercial stingless bee honey were randomly obtained from the local market around Malaysia.

All of the chemicals and reagents used were of analytical grade. Sugar standard (fructose, glucose, sucrose), acetonitrile, ethanol, sodium bisulfate, acetate buffer and sodium hydroxide (NaOH), were purchased from Sigma-Aldrich (USA). Carrez solution I and II were purchased from Merck (Germany). Sodium chloride and iodine were obtained from R&M (Malaysia). Plate count agar (PCA), violet red bile glucose agar (VRGB), Sabouroud Dextrose agar (SDA) and Mueller Hinton agar were purchased from Oxoid, UK. Finally, chloramphenicol was obtained from Nacalai Tesque (Japan).

Two strains of the gram-positive bacteria and gram-negative bacteria: *Escherichia coli* and *Bacillus sp.* used in this study were obtained from the Central Laboratory, University Malaysia Pahang. The isolates were identified based on standard microbiological techniques, and sub-cultured in nutrient agar slopes at 37 °C for 24 h.

Instrumentation

Instrumentation used were SevenCompact pH meter (Mettler Toledo, USA), Hand-held refractometer (RHB 90ATC, China), Hi 8733 conductivity meter (Hanna Instruments, USA), Carbolite CWF 1200 muffle furnace (Carbolite Gero Limited, UK), GENESYS 10S UV-Vis spectrophotometer (ThermoFisher Scientific, USA), LP vortex mixer (ThermoFisher Scientific, USA), 1260 Infinity II LC System (Agilent Technologies, USA), BS -21 shaking water bath (Lab Companion, Jaio Tech, South Korea) and Incubator I (Memmert, Germany).

Procedure

Physicochemical analyses

Determination of pH and moisture content. The pH of the honey sample was measured by diluting 10 g of

Table 1. Sampling location, description and time collection of six samples of stingless bee honey from Malaysia

Sample Code	Description	Sampling Location	Time of collection
H1	Harvested honey	Pahang	March 2017
H2	Harvested honey	Pahang	March 2017
H3	Commercial honey	Selangor	October 2017
H4	Commercial honey	Kedah	October 2017
H5	Commercial honey	Kuala Lumpur	November 2017
H6	Commercial honey	Pahang	September 2017

of honey with 75 mL of distilled water while moisture content was determined by dropping approximately 1 to 2 mL of honey samples to the measuring surface of the handheld refractometer.

Determination of free acidity. The free acidity was measured by diluting 10 g of honey in 75 mL of distilled water before this solution was titrated with 0.1 M NaOH solution. pH readings were observed simultaneously during titration until the pH reached 8.5, and the results were expressed in mmol/L.

Determination of electrical conductivity and ash content. The electrical conductivity was measured by diluting 20 g of honey in 100 mL distilled water (20% w/w) where the results were expressed in milliSiemens per centimeter (mS/cm). The honey ash content was determined by placing the crucible in an oven for 1 h. After cooling, the crucible was weighed, and 5 g of the honey sample was added into the crucible before burnt in a 500 °C furnace for 2 h until constant mass was obtained. The sample was then reweighed, and ash percentage was calculated.

Determination of hydroxymethylfurfural (HMF). Determination of HMF was carried out by following the International Honey Commission's harmonized methods [11] where 5 g of honey sample was weighed and completely dissolved in 25 mL of distilled water. The volumetric flask containing a honey solution was added with 0.5 mL Carrez solution I and mixed well by vortex. The mixed solution was then added with 0.5 mL Carrez solution II and mixed before adding distilled water up to 50 mL mark. A drop of ethanol was added to suppress the foam that formed during mixing. The first 10 mL of the mixture was disposed of after being filtered through filter paper. 5.0 mL of the solution was placed in two test tubes where in Tube 1 (sample solution) was added with 5.0 mL of distilled water and in Tube 2 (reference solution) was added with 5.0 mL of 0.2% sodium bisulfate solution. The absorbance of the solutions was recorded at 284 and 336 nm, respectively.

Determination of sugar profile. The determination of sugar (fructose, glucose, sucrose) were performed following the method of Malaysian Kelulut Standard [12]. The sugars were eluted through Phenomenex column (PhenoSphere 5μ NH_2 80A, 250 × 4.6 mm, Phenomenex Inc, USA) and detected by Refractive Index detector (RID) operated at 40 °C. The mobile phase is acetonitrile: water (80:20, v/v) at a flow rate of 1.3 mL/min. The retention times obtained from the standards were compared to obtain HPLC sample peaks. The injections were performed in triplicate where the average peak area was used for evaluation.

Determination of diastase activity. The diastase activity of honey was determined by dissolving 5.0 g of honey in 15 mL distilled water and 2.5 mL of acetate buffer (1.59 M, pH 5.3). The samples solution was then mixed with 1.5 mL of 0.5 M NaCl solution before 10 mL of this solution was transferred in a test tube containing 5 mL of 2% starch solution. The test tube was then kept in a water bath at 40 °C for 5 min, and 1 mL of the solution was added with 10 mL of 0.0007 M diluted iodine solution. The absorbance was recorded at 660 nm in spectrophotometer until readings showed absorbance less than 0.235. The diastase activity was expressed in Gothe degrees. DN was the amount of enzyme that hydrolyzed/converts 1% starch solution/0.01g of starch for 1 h at 40 °C.

Microbiological analyses

Standard plate count. The honey samples that were diluted in saline water were plated on standard plate count agar and incubated at 30 °C for 48 h.

Detection of *Bacillus sp*. The initial dilution containing the aerobic spore-forming bacteria was heated in 10 minutes at 80 °C and immediately immersed in cold water afterward to cool down the temperature. *Bacillus sp.*
was detected by plating the dilutions on plate count agar (PCA) and incubated at 30 °C for 48 h.

Detection of total coliform. The samples were plated on violet red bile glucose agar (VRGB) and incubated at 35 °C for 48 h.

Yeast and Moulds count. Yeast and Moulds were quantified after plating the samples on Sabouroud Dextrose agar (SDA) supplemented by 100 mg/L chloramphenicol. The plates were incubated at 25 °C for 5 days. Microbial counts were expressed as colony-forming units per gram of honey sample (cfu/g).

Antibacterial analysis: Agar disc diffusion method

The agar diffusion method was determined by following the method by Moussa [13]. Fresh culture suspension of the test microorganisms (100 µL) was spread on Mueller Hinton agar. The concentration of cultures was 1×10^7 CFU/mL. For screening, 5 mm sterile diameter filter paper disc was infused with 10 µL of honey equivalent to 0.1 mg of honey. The plates were placed at 4 °C for 2 h before being incubated under optimum conditions for 24 h. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. The zone diameters of inhibition (ZDI) was measured in millimeter, including the diameter of the disc. The controls were set up with equivalent quantities of water as a control.

Statistical analysis

All analyses were prepared in triplicate. The data obtained in the study were analyzed using analysis of variance (ANOVA) and followed by Tukey test (Minitab 18, Minitab Inc., USA) where the differences between mean values were significant at values of $p < 0.05$.

■ RESULTS AND DISCUSSION

Physicochemical Analyses

Table 2 reported the physicochemical properties of the different samples of honey obtained from a local market in Malaysia. According to the result, pH values ranged from 2.51 to 3.26 which met the criteria of pure honey. There was no significant difference in the pH values of H1 and H2 ($p > 0.05$). However, when compared with H3, H4, and H5, the results were significantly different. The pH values varied depending on their geographical origin, floral sources as well as the bee's species [14]. Honey from warm and humid countries usually has lower pH due to their high water content. The pH of adulterated honey is higher (more than pH 5.5) compared to pure honey due to the extraction, storage factor, and temperature of honey [7].

From the result, six selected honey samples showed that the percentage of moisture content fluctuated from 19.4 to 30.9% which is still in the range of Malaysian Kelulut standard. The variation may be due to the humidity of tropical forest, floral origin, soil, collection period and processing aspects [14-15]. H2 has the highest moisture content while H4 has the lowest moisture content. This may be due to the collection time of H2 which was during the rainy season while H4 may have undergone moisture removal. Besides increasing the probability of fermentation happening, higher moisture content (more than 35 %) can also indicate that the honey is adulterated [16-17].

Typically, a small amount of acid can be found in pure honey which is significant for a taste of honey [18]. From the result, H4 has the highest free acidity with a value of 318.7 meq/kg. There is no fixed limit of free acidity in Malaysian Kelulut standard, but all six samples

Table 2. Summary of physicochemical analyses of six sample of stingless bee honey from Malaysia (mean ± standard deviation, n = 3)

Sample	pH	Moisture (%)	F.A (meq/kg)	EC (mS/cm)	Ash (g/100g)	HMF (mg/kg)	Diastase Number (DN)	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)
H1	3.26±0.11^{a}	25.4±0.09^{d}	146.4±7.46^{b}	0.56±0.01^{b}	3.11±0.23^{a}	74.3±6.88^{b}	5.97±0.18^{a}	17.5±1.3^{a}	16.0±0.8^{b}	< 0.01
H2	3.20±0.08^{a}	30.9±0.06^{a}	121.1±1.24^{b}	0.33±0.01^{c}	0.72±0.11^{b}	85.9±8.40^{b}	5.85±0.08^{a}	15.03±1.22^{a}	12.17±0.47^{b}	< 0.01
H3	2.52±0.03^{c}	23.4±0.53^{e}	257.8±2.39^{a}	0.68±0.01^{a}	2.75±1.12^{a}	457.2±0.81^{a}	2.32±0.12^{b}	30.96±0.81^{b}	31.6±0.79^{a}	1.10±0.03^{a}
H4	2.79±0.01^{b}	28.4±0.51^{b}	318.7±77.6^{a}	0.40±0.03^{c}	3.86±1.02^{a}	35.4±0.71^{c}	6.30±1.16^{a}	32.52±0.59^{b}	30.09±0.70^{a}	< 0.01
H5	2.51±0.01^{c}	19.4±0.21^{f}	250.7±3.19^{a}	0.69±0.06^{a}	4.31±1.53^{a}	461.7±5.69^{a}	2.16±0.13^{b}	31.64±0.48^{b}	34.55±0.50^{a}	2.81±0.09^{a}
H6	2.57±0.01^{c}	26.7±0.61^{c}	241.7±3.46^{a}	0.65±0.04^{a}	0.52±0.16^{b}	456.6±2.72^{a}	2.79±0.01^{b}	22.83±0.42^{c}	25.47±0.50^{c}	7.29±0.18^{b}
Mean ± SD	2.81±0.34	25.7±4.01	222.7±74.5	0.55±0.15	2.54±1.58	261.8±216.1	4.23±2.0	25.08±7.70	24.98±9.02	3.73±3.20
Min Value	2.51	19.4	121.1	0.33	0.52	35.4	2.16	15.03	12.17	<0.01
Max Value	3.26	30.9	257.8	0.69	4.31	461.7	6.30	32.52	34.55	7.29

Note: $^{a-f}$=Means with a different superscript letter along the column are significantly different ($p < 0.05$); F.A: Free acidity; EC: Electrical conductivity; HMF: Hydroxymethylfurfural

acid values exceeded the international honey standard, which is no more than 50 meq/kg. This may explain the sour taste that the stingless bee exhibit. The lower value of acidity indicates the freshness of honey however the value may increase with time. This is due to the fermentation process where sugars converted into organic acids. [7,19]. Besides that, flower sources and bee species influence the variation of acidity value since it conforms to the balance of organic acids present in honey [15].

Electrical conductivity relies predominantly on the mineral content of honey [20]. From the six samples, H5 had the highest value of electrical conductivity with 0.69 ± 0.69 mS/cm which may indicate that the honey is rich in mineral content. The differences in electrical conductivity correspond to not only the different geographical and floral sources but also the number of organic acids, proteins and storage time [14,21]. The color of honey also influence the electrical conductivity values as dark honey gives higher conductivity due to higher levels of microelements than light honey [22].

From the result of ash, only H2 and H6 were observed to be within the range of Malaysian Kelulut standard (< 1.0 g/100g) while others reported being higher. The high value of ash may contribute by the floral source and nectar characteristic in some floral species [23]. There is a wide distribution of values detected in all six samples which may contribute by an irregular pattern of harvest processes and the different in meliponiculture techniques used by the producers [24]. Ash content and electrical conductivity are closely related to the mineral content in honey, but ash is differing as it directly measures the inorganic residue after carbonization [25].

HMF is one of the indicators used to assess the quality of honey which is absent in fresh honey [26]. Results showed that H4 has the lowest HMF values (35.4 ± 0.71 mg/kg) but still exceeded the limit set by the Malaysian Kelulut standard (< 30 mg/kg). The samples H3, H5, and H6, exceeded the HMF limit in both international standard and Kelulut standard (> 400 mg/kg) which suggest that the three honey samples have undergone a heating process or adulterated. Furthermore, high HMF content in honey also demonstrates the fabrication of honey with invert syrup since HMF can be formed by heating sugars in the presence of an acid to the inversion of sucrose [27-30]. Even though HMF of H1, H2, and H4 were quite high, the result may cause from poor storage condition, or the samples were old. The values of HMF could increase during processing, preparation, aging, and storage of honey [26].

In good quality honey, the fructose content should exceed the glucose content [16,31], except in three samples (H3, H5, and H6). Thus, H3, H5, and H6 probably had poor quality. Harvested honey samples, H1 and H2, had lower fructose and glucose content compared to other commercial samples but still comparable with the study of Thailand and Malaysian stingless bee honey [32-33]. Sucrose content should be within 8.0 g/100 g, and all analyzed samples were still within the Malaysian Kelulut standard limit. The high concentration of sucrose may due to the diversity of floral sources, early harvest of honey as the sucrose not fully transform into fructose and glucose, overfeeding the bees with sugars, syrups or artificial honey and lastly, the honey is adulterated by the addition of commercial sugar [14,34-35].

Denoted as Diastase Number (DN), diastase activity for six samples of honey ranged from 2.6 to 6.30 DN. There is no fixed limit for Diastase Number in Malaysian Kelulut standard, but according to international honey standard, Diastase Number should be no more than 3 for honey with low enzyme content. H3, H5, and H6 samples have low DN number (less than 3 DN) which may mean that these honey is aging or has been heated since enzymes are susceptible towards heat [36]. Diastase Number is not only controlled by geographical and botanical origin but also by pH values, nectar flow and foraging patterns of the bees [24-25].

Microbiological Analyses

The microbial counts of six stingless bee honey samples can be observed in Table 3. The standard plate counts (SPC) were found in every sample with a count of 1×10^2 cfu/g to 9.7×10^2 cfu/g. The total plate counts variation may be influenced by the honey characteristic, honey freshness, the harvest period and infection by pathogenic bacteria [6,25]. The most common types of

Table 3. Summary of the microbial profile of honey samples from Malaysia

Sample	Microbial count ($\times 10^2$ cfu/g)				
	SPC	*Bacillus sp.*	Total coliform	Yeast	Mold
H1	2.0×10^2 1.5×10^2 1.0×10^2	ND	D	ND	ND
H2	3.0×10^2 8.0×10^2 4.4×10^2	ND	ND	ND	ND
H3	3.6×10^2 4.5×10^2 1.5×10^2	D	D	D	D
H4	2.6×10^2 2.7×10^2 9.7×10^2	D	ND	D	D
H5	1.5×10^2 2.2×10^2 3.5×10^2	D	ND	D	D
H6	1.0×10^2 1.0×10^2 1.0×10^2	D	ND	ND	ND

SPC: Standard Plate Count; D = Detectable; ND=Not Detectable

Table 4. Summary of antibacterial activity of various honey samples on gram-positive and gram-negative bacteria

Sample	Dilution	*Bacillus sp.* (mm)	*E. coli* (mm)
H1	Undilute	15	11
	10^{-1}	-	-
	10^{-2}	-	-
H2	Undilute	24	11
	10^{-1}	-	-
	10^{-2}	-	-
H3	Undilute	20	-
	10^{-1}	-	-
	10^{-2}	-	-
H4	Undilute	-	-
	10^{-1}	-	-
	10^{-2}	-	-
H5	Undilute	12	-
	10^{-1}	-	-
	10^{-2}	-	-
H6	Undilute	10	-
	10^{-1}	-	-
	10^{-2}	-	-

Bacillus sp. in honey are *B. cereus, B. megaterium, B. coagulans, and B. pumilus* [25]. *Bacillus sp.* were absence in H1 and H2 which may indicate that the honey is well conserved against this bacteria. The symbiotic relationship

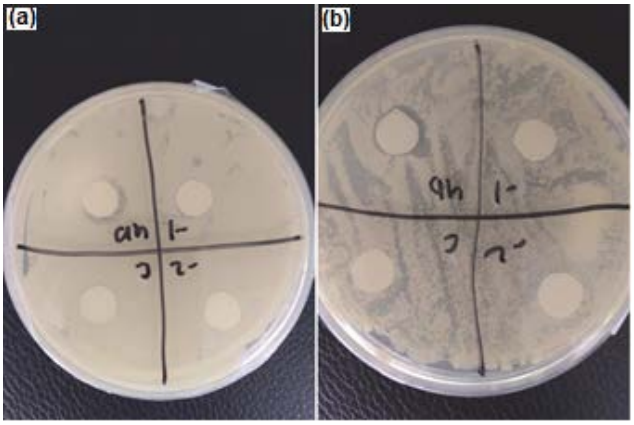

Fig 1. (a) The inhibition zone by using different dilution factor on *E. coli* (b) The inhibition zone by using different dilution factor on *Bacillus sp.*

between *Bacillus sp* and insects especially tropical honey bees and stingless bees may be the solid reason why these bacteria were present in the other four samples [37]. Total coliform indicates the sanitary quality of honey [21], and for this study, the coliform was present only in H1 and H3 samples. The low counts and limited variety of microbes are expected because of honey antibacterial properties against the growth or persistence of many organisms [31,38]. Yeast and mold were not detected for H1, H2, and H6 but, it was present in a high number (> 100) for the other three samples. The result for H3, H4, and H5 contradict the limit set by Malaysia Kelulut standard where the count should be less than 1×10^1 cfu/g.

Antibacterial Analysis: Agar Disc Diffusion

From Table 4, H1, and H2 had a greater inhibitory effect on both gram-negative (*E. coli*) and gram-positive bacteria (*Bacillus sp.*) when tested using undiluted honey. The results of commercial honey samples also showed they had an inhibitory effect only on gram-positive bacteria when tested using undiluted honey (except H4), but all the commercial honey samples were not active against gram-negative bacteria, *E. coli*. The disc diameter of harvested samples was ranged from 11 to 24 mm where commercial honey, H3, H5, and H6, ranged from 10 to 20 mm. There were no zone diameters of inhibition (ZDI) was measured on H4 sample for both bacteria tested. Among all honey samples, H1 and H2 are the only honey that have effects on gram-negative bacteria. Thus, the honey may have the potential as therapeutic or healing honey [13]. The antibacterial activity of honey depends on various factors such as geographical origin, botanical source, harvesting (season when honey was collected), processes and storage conditions as well as the presence of hydrogen peroxide, phenolic compounds, acidity, phytochemicals, pH of honey and higher osmotic pressure [13,25,39].

■ CONCLUSION

In this present work, six samples honey from the local market in Malaysia have been analyzed for their quality criteria concerning the physicochemical parameters and microbial profile. All analyzed samples are within the maximum limit of the quality criteria set by the Malaysian Kelulut Standard and Codex Alimentarius except for free acidity, HMF, and Diastase Number. Most samples have a high value of HMF (more than 400 mg/kg) and lower Diastase Number (lower than 3 DN) especially for H3, H5, and H6. These three samples may indicate poor honey processing and the possibility of adulteration. Even though H1 and H2 were purely harvested from the farm, the quality of both honey is quite poor which may be due to prolonged storage (more than six month) and the poor storage condition. There is no limit set by the Malaysian standard for free acidity, electrical conductivity, and Diastase Number. Hopefully, in future, these parameters can be added into the Malaysian Kelulut Standard to ensure the standard is as per with another international standard available. Honey is known for its antibacterial properties; however, microbial growth has been found in most samples, expressing poor hygienic procedures during harvesting, packaging or storage. Although this study was a preliminary work and only limited to six samples, more sample data are in preparation in order to be part of the process to create more specific legislation for stingless bees honey in Malaysia.

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