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Physicochemical and Proximate Analysis of *Heterotrigona itama* Honey from Inland and Coastal Regions of Sarawak

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This project aims to examine the biochemical properties of *Kelulut* honey produced by *Heterotrigona itama* from two different geographical origins in Sarawak. In the present study, the honey samples originated from inland region of Nanga Dap, Kanowit and coastal region of Tanjung Manis, Mukah of Sarawak, Malaysia were assessed and compared for their physicochemical and proximate properties. Ninety-six (96) samples from two different areas of Sarawak were evaluated and compared by using independent T-test of SPSS software. The results revealed significant correlations ($p < 0.05$) of moisture ($p = 0.002$), total phenolic content ($p = 0.007$), glucose content ($p = 0.000$), maltose content ($p = 0.005$), 5-hydroxymethylfurfural (5-HMF) ($p = 0.004$), pH ($p = 0.000$), protein ($p = 0.032$), acetic acid ($p = 0.032$), acetic acid ($p = 0.000$), gluconic acid ($p = 0.000$), ash ($p = 0.001$), carbohydrates ($p = 0.001$) and energy ($p = 0.001$) for honey samples from these two areas. On top of that, the honey samples from Tanjung Manis possess higher moisture content, total phenolic content, sucrose content, maltose content, acetic acid, gluconic acid and ash.

Keywords: *Heterotrigona itama* honey; Sarawak; physicochemical; proximate

I. INTRODUCTION

In view of its medicinal and therapeutic characteristics, honey is one of the nutritious food products and valuable supplements with growing interest among the healthy population. Honey exhibits biological properties such as cancer chemopreventive agent, antiseptic, anti-inflammatory and antimicrobial (Alvarez-Suarez *et al.*, 2012; Badolato *et al.*, 2017; Da Silva *et al.*, 2013; Vit & Tomás-Barberán, 1998). Despite the relevant importance of antioxidant activity in food, honey such as stingless bee honey is regarded as a health-promoting food which possesses abundant source of phenolic compounds (Da Silva *et al.*, 2013; Kek *et al.,* 2014; Sousa *et al.*, 2016; Tuksitha *et al.*, 2018) and polyphenolic compounds are known as one of its major constituents (Da Silva *et al.*, 2013; Sousa *et al.*, 2016).

The originality of honey is associated with bee origin. In addition, the physicochemical and phytochemical compositions of the honey are greatly dependent on the geographical origins, seasonal, environment factors and floral source (Alvarez-Suarez *et al.*, 2010; Badolato *et al.*, 2017; Escuredo *et al.*, 2014; Kek *et al.*, 2017; Sousa *et al.*, 2016). Honey is normally produced by both honeybees (mainly the *Apis* species) and stingless bees. Over six hundred species of stingless bees have been found in subtropical and tropical parts of the world, for example, South America, Africa, Southeast Asia and Australia (Boorn *et al.*, 2010; Michener, 2013).

Research on the compositions of stingless bee honey in different geographical origins such as Brazil (Biluca *et al.*,

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2016; Da Silva *et al.*, 2013; Sousa *et al.*, 2016), Thailand (Chuttong *et al.*, 2016), Malaysia (Kek *et al.*, 2014; Moniruzzam *et al.*, 2014; Tuksitha *et al.*, 2018) had been reported. The variability of chemical compositions and characteristics of honey are dependent on the bee species and botanical origin. As an example, the moisture content of stingless bee honey samples from Brazil was lower (23.1% to 43.5% w/w) in comparison with samples from Thailand (25% to 47% w/w) as reported by Biluca *et al.* (2016) and Chuttong *et al.* (2016), respectively. Other than that, Oddo *et al.*, (2008) also revealed that moisture content, electric conductivity and free acidity of stingless bee (*Tetragonula carbonaria*) honey samples were higher than the honey harvested from *Apis mellifera* species.

The increasing production of stingless bee honey and overwhelming responses of market for natural remedies necessitate the need of research on composition of honey from different geographical origins and thus reflect the originality of the honey products. Meanwhile, there is a limitation of identity and quality standards on the stingless bees' product (Guerrini *et al.*, 2009). Standards set by the Codex Alimentarius Commission (2001) could not be applied for the stingless bee honey as this standard is specifically apply to the *Apis mellifera* honey. At this moment, Standards Malaysia (2017) is the main reference point for checking of the physiochemical and nutritional properties of the stingless bee honey produced in Malaysia.

The principle objective for this study is the physiochemical properties examination of the stingless bee honey from apiaries of two different geographical origins in the state of Sarawak where past research efforts have been minimal. The honey samples were collected from bee farms which are located at Nanga Dap (inland region) and Tanjung Manis (coastal region) of Sarawak, Malaysia (Figure 1).

Figure 1. Location of Nanga Dap (inland region) and Tanjung Manis (coastal region) of Sarawak

Several analyses such as moisture content, total phenolic content, predominant sugars content, 5-hydroxymethyfurfural (5-HMF), acetic acid, gluconic acid, pH, ash, energy, protein, carbohydrates and energy were conducted. Following this, an independent T-test was employed using SPSS software to determine the correlation between the data from the two different geographical origins.

II. MATERIALS AND METHODS

A. Honey Samples

Raw stingless bee honey samples were provided by Rimbunan Hijau Bee Farms Sdn. Bhd, Sibu, Sarawak, Malaysia. The bee farms are located at the uphill area of Nanga Dap, Kanowit, Sarawak, Malaysia and coastal region of Tanjung Manis, Mukah, Sarawak, Malaysia as shown in Figure 1. The total of ninety-six (96) honey samples were harvested in November 2017 (Table 1).

Table 1. Honey sample from two different geographical origins

Species	**Geographical origins (Sarawak, Malaysia)**	**Types of regions**	**Number of honey samples, *n***
H. itama	Nanga Dap, Kanowit	Inland area	45
	Tanjung Manis, Mukah	Coastal area	51

The stingless bee honeys were produced by *Heterotrigona itama* (*H. itama*) species where nectar sources are mainly from *Acacia mangium* trees. The honey samples were extracted by using laboratory syringe from independent honey hives and inserted into individual glass bottles. All the samples were refrigerated at 4°C until analysis within 2 weeks.

B. Sample Analysis

1. Physicochemical Analysis

In physicochemical analysis, six types of chemical properties were investigated – moisture content, total phenolic content (TPC), sugars content, 5-Hydroxymethylfurfural (5-HMF), pH and organic acids.

a. Moisture Content

The moisture content of honey samples was measured by using refractometer (RHF-30ATC, China) according to AOAC Official Method 919.38 (AOAC, 2016). All measurements were performed in 20°C.

b. Total Phenolic Content (TPC)

The TPC was examined using Folin Ciocalteu spectrophotometric method (Kek *et al*., 2014). The honey sample (1 g) was diluted to 20 mL with distilled water. The honey solution (1 mL) was then pipetted into 5 mL of Folin Ciocalteu reagents (0.2 N) and incubated for 5 min in room temperature. Then, 4 mL of 7.5% w/v aqueous sodium carbonate solution was added and further incubated at room temperature for 2 h. The absorbance of the mixture was measured at wavelength 765 nm against distilled water blank by using the UV-VIS spectrophotometer (Cary 60, Agilent Technologies, U.S.A). Gallic acid was used to produce the standard calibration curve with concentration ranging from 20 to 100 ppm. The total phenolic content was expressed in mg of gallic acid equivalent (GAE) per kg of honey.

c. Sugars Content

The predominant sugars content (fructose, glucose, maltose and sucrose) of stingless bee honey were determined according to AOAC Official Method 977.20 (AOAC, 2016) by using high performance liquid chromatography (HLPC) method. One gram of honey was dissolved in 20 mL of distilled water, filtered with 0.45 µm filter paper followed by injection into HPLC 1200 Infinity LC system (Agilent Technologies, U.S.A) which was equipped with autosampler and Evaporating Light Scattering Detector (ELSD). The analytical column used was Agilent Zorbax Carbohydrate (150 × 4.6 mm, 5 µ) while mobile phase is the mixture of acetonitrile and water (HPLC grade) in the ratio of 75:25 was used at flow rate 1.4 mL/min with oven temperature 30°C. High purity sugars standards with known concentrations were used to produce standard calibration curves with concentration ranging from 2 to 10 g /L. The sugar content was expressed in g per 100 g of honey.

d. 5-Hydroxymethylfurfural (5-HMF) Analysis

Determination of 5-HMF was conducted according to AOAC Official Method 980.23 (AOAC, 2016) by using the HPLC 1200 Infinity LC system (Agilent Technologies. U.S.A) equipped with an autosampler and a photodiode array detector. A 5% w/v of honey solution was prepared and filtered through 0.45 µm nylon filter. Isocratic elution was performed on a ZORBAX Eclipse XDB C18 column size 4.6 × 150 mm, 5 µm (Agilent Technologies, U.S.A) and mobile phase methanol-water (10:90, v/v) at flow rate of 0.5 mL/min. The injection volume was 20 µL, column temperature of 25°C and at wavelength of 280 nm (Mendes *et al*., 1998). The standard calibration curve was generated with concentration of 5-HMF from 5 to 25 mg/kg. The amount of 5-HMF was recorded in the unit mg per kg of honey.

e. pH Analysis

The pH of honey samples was determined according to AOAC method 962.19 (AOAC, 2016). A waterproof H160 pH meter (Hach, USA) was used to measure the pH value.

f. Organic Acids Analysis

The two organic acids, gluconic acid and acetic acid, of honey samples were identified and quantified by using HPLC method with minor modifications (Cherchi *et al*., 1994). One gram of stingless bee honey sample was dissolved in 20 mL of distilled water, filtered with 0.45 µm filter paper followed by injection into HPLC system equipped with photodiode array detector. The identification of gluconic acid and acetic acid was performed by isocratic elution with reversed phase column, ZORBAX Eclipse XDB C18 (Agilent Technologies, U.S.A) and ion-exclusion column, Phenomenex Rezek ROA-Organic acid column (Phenomenex, U.S.A), respectively. The experiments were

conducted with the mobile phase of 0.005 N sulphuric acid and flow rate of 0.5 mL/min. The injection volume was 10 µL with the column temperature of 40°C and the 210 nm of wavelength. The organic acids standard curves were prepared for concentrations ranging from 2 to 10 g/L each. The organic acids were expressed in percentage (%) of each compound.

2. Proximate Analysis

In proximate analysis, the energy value, carbohydrates content, crude protein and ash were evaluated. The analyses were performed according to AOAC, 2016.

a. Energy Value

The energy value was calculated using (1)

Energy Value (in kcal per 100 g honey)
$$= [(\text{Protein} \times 4) + (\text{Total Carbohydrate} \times 4) + (\text{Fat} \times 9)] \quad (1)$$

b. Carbohydrates

The carbohydrates contents were calculated using (2).

$$\text{Carbohydrates (in unit g/100 g)} = 100 - [\text{Moisture} + \text{Ash} + \text{Fat} + \text{Protein}] \quad (2)$$

c. Crude Protein

The Kjeldahl method according to AOAC 920.52 (AOAC, 2016) was applied in determination of crude protein content. The honey sample (2 g) was digested with digester machine (FOSS Labtec Line, Sweden) equipped with scrubber at 420°C for 1 h 15 min. After cooling, digester tube undergone distillation process with distillation machine (FOSS Kjeltec 8100, Sweden). Then, titration step was done by using 0.1 N of hydrochloric acid (HCl) with receiver solution consists of methyl red and bromocresol green as indicator in 0.4% boric acid. Percentage of protein was calculated from the percentage of nitrogen content (3) with universal conversion factor of 6.25 (4).

$$\%N = \frac{[(\text{Normality of HCl}) \times (\text{Volume sample} - \text{Volume blank}) \times 0.014 \times 100]}{\text{Weight of sample}} \quad (3)$$

$$\% \text{ Protein} = \%N \times 6.25 \quad (4)$$

d. Ash Content

Ash content of honey samples was measured according to AOAC Official Method 920.181 (AOAC, 2016) by placing crucible at 100°C in an oven for one hour. After cooling in desiccator, the weight of empty crucible was measured. Then, five grams of honey was placed into a crucible and then incinerated at 600°C for 2 h in furnace (Nabertherm, Germany). The weight of the crucible was measured again after cooling in desiccator.

3. Statistical Data Analysis

Statistical analysis was carried out with IBM Statistical Package for Social Sciences (SPSS) (SPSS Inc, U.S.A) version 23. Independent T-test was conducted in order to evaluate the significant difference at confidence level of 95% ($p < 0.05$) between the quantified physicochemical and proximate compositions of the stingless bee honey samples from the two different geographical origins.

III. RESULTS AND DISCUSSION

A. Physicochemical Analysis

Table 2 shows the average results of physicochemical and proximate properties in mean ± standard deviation. The moisture content of the stingless bee honey originated from Nanga Dap was 30.35 ± 1.47% while moisture content of samples from Tanjung Manis was 31.25 ± 1.40%, respectively.

Table 2. Physicochemical properties of Heterotrigona itama honey samples from two different geographical origins

Honey origins	**Nanga Dap (*n* = 45)**	**Tanjung Manis (*n* = 51)**
Moisture (%)	30.35 ± 1.37[a]	31.25 ± 1.40[b]
Total phenolic content (mg GAE/kg)	435.38 ± 133.86[a]	509.20 ± 126.70[b]
Fructose (g/100 g)	22.68 ± 3.34[a]	21.56 ± 3.44[a]

Glucose (g/100 g)	24.77 ± 3.53 [a]	22.47 ± 2.47[b]
Sucrose (g/100 g)	0.04 ± 0.18[a]	0.04 ± 0.30[a]
Maltose (g/ 100 g)	24.89 ± 10.07[a]	29.57 ± 5.63[b]
5-HMF (mg/kg)	0.91 ± 1.88[a]	0.11 ± 0.37[b]
pH	3.38 ± 0.23[a]	3.23 ± 0.09[b]
Gluconic acid (%)	7.83 ± 1.25[a]	9.62 ± 1.27[b]
Acetic acid (%)	0.16 ± 0.12[a]	0.36 ± 0.19[b]

Mean values in the same column with different superscript letters are significantly different ($p < 0.05$).

Similar results were also reported by Chuttong et al. (2016) who analysed the moisture content of twenty-eight stingless bee honey samples and the average value was 31 ± 5.4 g/100 g. The stingless bees' colonies are predominantly found in the tropical and subtropical regions of the world (Guerrini *et al.*, 2009). Tropical areas which include rainforests have abundant rainfall and high humidity and hence contribute to the higher moisture content of stingless bee honey. In comparison with the normal stinging honeybee *Apis mellifera*, the stingless bee honey are normally with higher moisture content. For example, the moisture content of all tested Saudi Apis mellifera honeys was found ranging from 12.12% to 17.32% (Alqarni *et al.*, 2012).

From the present study, the moisture content of honey sample from Nanga Dap is significantly lower ($p < 0.05$) as compared to honey sample from Tanjung Manis.

The location of the bee farms, one in inland while the other at coastal areas, as well as harvesting period of honey samples in November are believed to be the two main reasons for the significant differences in moisture content of honey samples. In view of atmospheric situations, conditions widely vary from inland due to land sea interface, temperature contrast, and the consequent development of local circulation (Yerramilli *et al.*, 2008). In addition, the climate variability in Sarawak is very prominent during Northeast (NE) monsoon which usually occurs between November and March (Sa'adi *et al.*, 2017) and the sudden surge of rainfall amount during monsoon might contributed to the higher moisture content for honey from Tanjung Manis. The average total phenolic content for honey sample from both inland and coastal regions of Sarawak were significant difference ($p < 0.05$), that is, 435.38 ± 133.86 mg GAE/kg and 509.19 ± 126.70 mg GAE/kg, respectively. The total phenolic content for samples from Tanjung Manis, Sarawak was higher compared to samples from Nanga Dap, Sarawak. The existence of polyphenolic compounds in honey are directly related to botanical resources and floral origins (Aljadi & Kamaruddin, 2004; Khalil *et al.*, 2011). Da Silva *et al.* (2013) reported the presence of fourteen (14) different phenolic compounds in methanol extracts of stingless honey samples. The polyphenolic compounds present in honey are normally from the nectar of flowers, pollen and propolis (Estevinho *et al.*, 2008; Da Silva *et al.*, 2013). In addition, the total phenolic content may become a significant indicator of the antioxidant capacity of honey samples (Khalil *et al.*, 2011 & Da Silva *et al.*, 2013). In the present study, the honey samples from coastal area, Tanjung Manis were with higher total phenolic content and hence with higher antioxidant capacity in comparison with the samples collected from the inland, Nanga Dap.

The average carbohydrate composition for Nanga Dap honey samples was fructose: 22.67 ± 3.34 g/100 g, glucose: 24.77 ± 3.53 g/100 g, sucrose: 0.04 ± 0.18 g/100 g and maltose: 24.89 ± 10.07 g/100 g. Meanwhile, the average carbohydrate composition for Tanjung Manis honey samples was fructose: 21.56 ± 3.44 g/100 g, glucose: 22.47 ± 2.47 g/100 g, sucrose: 0.04 ± 0.30 g/100 g and maltose: 29.57 ± 5.63 g/100 g. The honey samples from Tanjung Manis contain significantly higher content of maltose and glucose ($p < 0.05$) as compared to the inland area of Sarawak, Nanga Dap. The results of present study indicated that the local stingless bee honey samples were with lower monosaccharides and higher maltose content in comparison to those reported previously, such as, northwest Tunisian honey samples (Boussaid *et al.*, 2014) and Colombian stingless bee honey (Fuenmayor *et al.*, 2013). The simple sugars such as fructose and glucose were reported lower in stingless bee honey samples as compared to Apis mellifera honey samples (Chuttong *et al.*, 2016). The content of sucrose of honey samples from both regions of Sarawak was undetectable. Similar result was reported by Chuttong *et al.* (2016) when only five out of the twenty-eight stingless bees honey samples were detected with sucrose. Kek *et al.* (2017), however, detected 32.30 ± 2.66 g/100 g of sucrose from Kelulut honey samples under their investigation.

Significant correlation ($p < 0.0001$) was observed in

between the pH value and amount of organic acids (gluconic acid and acetic acid) of the stingless bee honey samples. The percentage of acetic acid and gluconic acid for honey samples from Tanjung Manis were higher in comparison with Nanga Dap honey samples. In the present study, the percentage of gluconic acid in *Heterotrigona itama* honey samples was found significantly high, that is, 9.62 ± 1.27% and 7.83 ± 1.25% for samples from Tanjung Manis and Nanga Dap, respectively. On the other hand, small amount of acetic acid was observed in the honey samples from both geographical origins. The present results support that the predominant acid in honey is gluconic acid (Karabagias *et al.*, 2014). Gluconic acid is formed by glucose oxidase which produced during the ripening process of honey. The higher amount of organic acids in Tanjung Manis honey samples caused the lower pH value of the samples. From the results, the average pH value was 3.38 ± 0.23 for Nanga Dap honey samples, and, 3.23 ± 0.09 for Tanjung Manis honey samples. According to Mato *et al.* (2006), organic acids are related to the chemical properties of honey such as acidity. A similar range of pH value (3.1 to 3.9) was reported for the twenty-eight (28) honey samples from eleven (11) stingless bee species originated from Thailand (Chuttong *et al.*, 2016).

The 5-HMF is normally used as indicator for the freshness of the honey samples which can be affected by heating or ageing during storage (Mendes *et al.*, 1998; Gomes *et al.*, 2011). According to Standards (2017), the level of 5-HMF should not exceed 30.0 mg per kg of honey. Meanwhile, the maximum proposed value of HMF by Codex (2001) is 80 mg/kg. In the present study, the mean value of 5-HMF for honey samples from Nanga Dap and Tanjung Manis were 0.91 ± 1.88 mg/kg of and 0.11 ± 0.37 mg/kg, respectively. The low level of 5-HMF in raw *Heterotrigona itama* honey indicated that the honey samples were very fresh. However, there were significant difference ($p < 0.05$) in the 5-HMF level for the honey samples from both inland and coastal areas. The higher 5-HMF level in Nanga Dap's honey samples might be due to the higher temperature and lower ventilation of certain beehives.

B. Proximate Analysis

Four types of proximate analysis were conducted in this study (Table 3).

Table 3. Proximate properties of *Heterotrigona itama* honey samples from two different geographical origins

Honey origins	**Nanga Dap (*n* = 45)**	**Tanjung Manis (*n* = 51)**
Crude protein (%)	0.42 ± 0.24[a]	0.34 ± 0.09[b]
Ash (%)	0.23 ± 0.19[a]	0.34 ± 0.10[b]
Carbohydrates (g/100 g)	7.89 ± 5.38[a]	4.50 ± 4.15[b]
Energy (g/100 g)	69.00 ± 1.35[a]	68.07 ± 1.42[a]

Mean values in the same column with different superscript letters are significantly different ($p < 0.05$)

The honey samples were evaluated on the ash, protein, carbohydrates and energy. Significant differences ($p < 0.05$) were observed for all the proximate parameters in honey samples originated from Nanga Dap, Sarawak and Tanjung Manis, Sarawak. The honey samples originated from inland of Sarawak, Nanga Dap, exhibited significantly higher protein, carbohydrates and energy values.

C. Statistical Analysis

The present study encompassed independent T-test for the statistical analysis using the SPSS software version 23. Table 4 shows the *t*-value, degree of freedom (*df*) and significance *p* value. From the results, significant difference ($p < 0.05$) was observed for all the parameters except the fructose and sucrose content. Positive *t* value indicates there was positive relationship between data with null hypothesis whereas the negative *t*-value indicated the vice versa. The *t*-distribution had 94 degree of freedom corresponds to the sample size of 96 in two- samples independent *t*-test.

Parameters	*t*-value	df[a]	Sig[b] (2-tailed)
Moisture	– 3.202	94	0.002
Total Phenolic Content	– 2.774	94	0.007
Fructose	1.597	94	0.114*
Glucose	3.735	94	0.000
Sucrose	– 0.044	94	0.965*
Maltose	– 2.858	94	0.005
5-HMF	2.987	94	0.004
pH	4.193	94	0.000
Protein	2.182	94	0.032
Acetic acid	– 6.081	94	0.000
Gluconic acid	– 6.960	94	0.000
Ash	– 3.473	94	0.001
Carbohydrates	3.293	94	0.001
Energy	3.556	94	0.001

Table 4. Independent T-Test (equal variances assumed)

Df[a] = Degree of freedom; Sig[b] = Significance

** not significant (p > 0.05)*

IV. CONCLUSION

There was significant difference ($p < 0.05$) between the physicochemical and proximate properties of *Heterotrigona itama* honey samples originated from inland and coastal regions of Sarawak. Stingless bee honey samples from Nanga Dap, inland region of Sarawak was found with significantly higher in glucose content, 5-HMF, pH value, protein, carbohydrates and energy. Therefore, the physicochemical and proximate properties of the stingless bee honey were dependent on the geographical regions of Sarawak.

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