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Evaluation of Physicochemical Properties of *Trigona sp.* Stingless Bee Honey from Various Districts of Johor

(Kajian fizikokimia terhadap *Trigon sp*. Madu Lebah Kelulut di Daerah Johor)

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ABSTRACT

Johor as one of the states of Malaysia is a good geographic location for meliponiculture activity. In this study, ten samples of stingless bee honey from each regions of Johor and one sample obtained from Institute Bioproduct Development, Universiti Teknologi Malaysia, Skudai were analyzed for the physicochemical properties of stingless bee honey. The physicochemical analyses were including moisture, total soluble solids, ash, pH, free acidity, conductivity, hydroxymethylfurfural (HMF) content, protein, carbohydrate, fat, dietary fibre, total phenolic content (TPC), total flavonoid content (TFC), and DPPH radical scavenging activity. Trigona sp. sample from Kluang have the highest value of phenolic content (778.23 ± 2.011 mg GAE/100 g) while Trigona sp. sample from Mersing have the highest value of flavonoid content (194.98 ± 0.350 mg RE/100 g). Among eleven samples tested for radical scavenging activity, Trigona sp. sample from Kota Tinggi have the highest scavenging activity (23.37 ± 0.36 mg/ml), but the value of HMF content exceeded the limit of Malaysian standard for stingless bee honey. All parameters were significantly different ($p < 0.01$) except for ash. This study showed a strong correlation between moisture and acidity ($r = 0.601$). However, low correlation was obtained between TPC and TFC with DPPH radical scavenging activity ($r = -0.235, 0.011$). The data obtained from this study could help for a better subsequent of Malaysian stingless bee honey industry.

Keywords: Trigona sp.; honey; physicochemical; hydroxymethylfurfural; antioxidant; DPPH

ABSTRAK

Johor sebagai salah satu negeri di Malaysia adalah lokasi geografi yang baik bagi menjalankan aktiviti meliponikultur. Dalam kajian ini, sepuluh sampel madu lebah kelulut dari setiap daerah di Johor dan satu sampel yang diperoleh dari Institut Pembangunan Bioproduk, Universiti Teknologi Malaysia, Skudai dianalisis bagi sifat fizikokimia madu lebah kelulut. Analisis fizikokimia adalah termasuk kelembapan, jumlah pepejal yang larut, abu, pH, keasidan bebas, konduktiviti, kandungan hidroksimetilfurfural (HMF), protein, karbohidrat, lemak, serat pemakanan, jumlah kandungan fenolik, jumlah kandungan flavonoid, dan aktiviti pemerangkapan radikal DPPH. Sampel Trigona sp. dari Kluang mempunyai kandungan fenolik tertinggi (778.23 ± 2.011 mg GAE / 100 g) manakala sampel Trigona sp. dari Mersing mempunyai kandungan flavonoid tertinggi (194.98 ± 0.350 mg RE / 100 g). Antara sebelas sampel yang diuji untuk aktiviti pemerangkapan radikal, sampel Trigona sp. dari Kota Tinggi mempunyai aktiviti pemerangkapan tertinggi (23.37 ± 0.36 mg / ml), tetapi nilai kandungan HMF melebihi had piawaian Malaysia untuk madu lebah kelulut. Terdapat perbezaan antara keseluruhan faktor fizikokimia ($p < 0.01$) kecuali faktor abu. Kajian ini menunjukkan korelasi yang kuat antara kelembapan dan keasidan ($r = 0.601$). Walau bagaimanapun, korelasi yang rendah diperoleh daripada jumlah kandungan fenolik dan jumlah kandungan flavonoid dengan aktiviti pemerangkapan radikal DPPH ($r = -0.235, 0.011$). Data yang diperoleh daripada kajian ini dapat membantu industri madu lebah Malaysia dalam kajian seterusnya.

Kata kunci: Trigona sp.; madu, fizikokimia; hidroksimetilfurfura; antioksidan; DPPH

INTRODUCTION

Honey has functional usage of being a good food sweetener and proposing high medicinal effects especially on human health. It can be used as home remedies such as honey mask, to cure flu and other medicinal effects. There are various types of honey in the world and mostly collected from the beehive of *Apis mellifera sp*. from genus *Apis* bees. At a certain years back in 1860s, researchers found that honey can also be collected from a type of bee named stingless bees.

Stingless bees, a type of bee which is stingless, are smaller than *Apis* bees (*Apis mellifera*, *Apis cerana*, *Apis dorsata*, etc.). Although being smaller, they can produce honey by collecting nectar from floral plants (do Nascimento et al. 2015) and transform to honey through enzymatic process even though the production are lesser than *Apis* bees. Other than being small, stingless bees could be differentiated with *Apis* bees by having a pot-like structure of honey pot instead of vertical honeycomb. There are about 500 species of stingless bees found across the world. They are most commonly found in tropical and sub-tropical countries like Africa, America, Australia and parts of Asia including Malaysia (Vijayakumar and Jeyaraaj 2014; Kek et al. 2014).

In Malaysia, more than 30 species of stingless bees from genus *Trigona* were found. The most popular species for rearing of the bees and having commercial values are *Geniotrigona thoracica* Smith, *Heterotrigona itama* Cockerell, *Lepidotrigona Terminata* Smith, *Tetragonula fuscobalteata* Cameron and *Tetraponera Laeviceps* (Kelly et al. 2014). Unlike *Apis* bees, the rearing of stingless bees in Malaysia is in early phase. In Johor, the rearing of stingless bees is growing increasingly in every regions of Johor. Therefore, there is high demand on the stingless bee honey in Johor.

Stingless bee honey and all types of honey are composed of carbohydrate, water, protein, minerals, vitamins and antioxidants that dependent on the floral types used, climatic changes and its maturation process (Rebiai and Lanez, 2014). Identification of the characteristics of honey is important to determine the quality of honey especially stingless bee honey as this honey are already available in Malaysian markets. Only recently there is a concern on the physicochemical characteristics of stingless bee honey in Malaysia for developing a regulatory standards.

Malaysian Standard (2017) released a standard specifically for Malaysian stingless bee honey (MS 2683: 2017) which stated the quality requirements of stingless bee honey. In this standard, the quality of raw stingless bee honey must followed these requirements: moisture content < 35%; sucrose content < 7.5%; ash content < 1.0%; hydroxymethylfurfural (HMF) content < 30 mg/kg; pH between 2.5 to 3.8; and presence of plant phenolics. The objective of this study was focusing on the determination of physicochemical characteristics of stingless bee honey from various regions in Johor, hence, obtained results were compared with present Malaysian standard.

MATERIALS AND METHODS

SAMPLE COLLECTION

A total of eleven stingless bee honey (n = 11) samples, each consisting of 200 g of honey, were obtained from beekeepers from various regions in Johor (Table 1). All honey were obtained in a period of three month from August 2016 until October 2016. The honey samples were obtained regardless of their species. The samples were tightly sealed in glass bottles and stored in the refrigerator (4 - 6 ºC) and kept at room temperature (27 ± 2 ºC) overnight before analyses were conducted. All samples were analyzed triplicate.

TABLE 1. Sources of samples collected from regions in Johor

Honey Code	Geographic regions of Johor stingless bee honey
TMU (Muar)	Kampung Jayor, Pagoh, Muar, Johor
TSG (Segamat)	Taman Yayasan, Segamat, Johor
TBP (Batu Pahat)	Taman Pantai, Batu Pahat, Johor
TJB (Johor Bahru)	Taman Kota Masai, Pasir Gudang, Johor
TKL (Kluang)	Felda Kahang Timur, Kluang, Johor
TKT (Kota tinggi)	Felda Semangar, Kota Tinggi, Johor
TMS (Mersing)	Felda Tenggaroh 2, Mersing, Johor
TKU (Kulai)	Kulaijaya, Johor
TP (Pontian)	Kampung Maju Jaya, Pekan Nenas, Pontian, Johor
TLD (Ledang)	Ledang, Johor
TIBD	Institute of Bioproduct Development (IBD), UTM Johor Bahru, Johor

PHYSICAL AND PROXIMATE ANALYSES

The pH value of honey samples were measured using a pH meter and the values were determined by directly measured into the honey samples. The moisture content was measured based on the refractometric method (AOAC Method 969.38) (AOAC 2000). A hand-held Refractometer (Atago, Japan) was used to determine the water. All measurements were expressed in percentage (% w/w).

The total soluble solid was measured by using a digital refractometer at (room temperature) (Saxena et al. 2010). The readings were corrected for a standard temperature of 20ºC by adding the correction factor of 0.00023/ºC. The analysis was carried out in triplicate and the results were expressed in ºBrix. The solution containing 20% (w/v) of honey was measured by using a conductivity meter (Camlab Water Model CW/6220, United Kingdom) to determine electrical conductivity of honey. All honey samples were analyzed and the results were reported in mS/cm. Two grams of honey samples was dissolved in 25 mL of water and homogenized, and the initial pH was recorded. The solution was titrated with 0.05 M sodium hydroxide (NaOH) until pH 8.50 to determine the free acidity. A 10 mL of 0.05 M NaOH was immediately added and the solution was titrated with hydrochloric acid (HCl) (0.05 M) until pH 8.3 to determine lactone acidity. The free, lactone and total acidity was calculated respectively. Results were expressed in mEq/kg.

Proximate analyses of honey including ash, protein, carbohydrate, fat and dietary fibre was conducted based on the methods of official analysis (Association of Analytical Chemists) (AOAC 2000). The ash content was determined by weighing (5-10 g) the honey samples in crucibles and placed into a muffle furnace at 600ºC to a constant weight (AOAC Method 920.181, 2000). Then, honey samples were cooled in desiccators until constant weight. The determination of ash was carried out in triplicate and the results were expressed in weight percentage (%). The protein content was determined by Kjeldahl method based on the total nitrogen content from the AOAC Official Method 991.20 (2000). Carbohydrate content was calculated based on Equation 1 (Chua and Adnan 2014).

$$\textit{Total carbohydrate}\left(\frac{g}{100g}\right) = 100 - (\textit{protein} + \textit{ash} + \textit{water} + \textit{fat} + \textit{dietary fiber}) \quad (1)$$

BIOCHEMICAL ANALYSIS

HYDROXYMETHYLFURFURAL (HMF)

HMF content was determined by using high performance liquid chromatography according to Rahaman (2014). A 2.5 g of honey was dissolved in 5 mL of water and topped up to 100 mL. The honey solution was filtered by using 0.45 µm nylon filter. A serial of standard 5-hydroxymethylfurfural solution (0-0.1 mg/ml) was used for calibration. Honey and standard solutions were injected to a high performance liquid chromatography (Waters 2690) coupled with a photodiode array detector (Waters 996). The C18 Synergy column (Synergy 4u fusion-RP 80A, 150 x 4.60 mm, 4 µm) was used for the separation. The mobile phase condition was; isocratic at 90% water and 10% methanol with 1% acetic acid; flow rate, 1 mL/min; injection volume, 20 µL. The chromatogram was monitored at 285nm. The concentration of HMF was determined from the calibration curve of standard HMF. The results were expressed in milligram per kilogram of honey (mg/kg).

TOTAL PHENOLIC CONTENT (TPC)

Folin-Ciocalteu method was used to determine the total phenolic content (Rahaman, 2014). A 0.5 (0.1 g/mL) mL of honey solution was mixed with Folin-Ciocalteu reagent (2.5 mL) (2 N). The mixture of honey and reagent was i mLcubated for 5 minutes. Then, the mixture was incuated for 2 hours after the addition of 2 mL of sodium carbonate ($NaCO_3$) (75%). Methanol was used as blank and the samples were measured at 760 nm by using UV-Vis spectrophotometer. Concentration between 0-100 mg/L of gallic acid was used to plot the standard calibration curve. The measurement was carried out in triplicate and presented in mg of gallic acid equivalents (GAE)/100 g of honey.

TOTAL FLAVONOID CONTENT (TFC)

The total flavonoid content determibation was conducted according to Rahaman (2014). A 5 mL of 2% aluminium chloride ($AlCl_3$) was mixed with 5 mL of honey solution (0.1 g/mL). After 10 minutes of incubation, the mixture of honey-reagent solution was measured at 415 nm by using a UV-Vis spectrophotometer (Perkin Elmer, Lambda 25). Concentration of rutin chemical from 0-100 mg/L was used as standard chemical for standard calibration curve of total flavonoid content. The measurement was performed in triplicate and presented in mg of rutin equivalents (RE)/100g of honey.

ANTIOXIDANT ANALYSIS

DPPH FREE RADICAL SCAVENGING ACTIVITY

The scavenging activity of free radical was determined using DPPH assays as described by Rahaman (2014). An amount of honey solution with range concentration between 1.5 to 170.0 mg/mL methanol was mixed with 1.5 mL of DPPH solution (0.02 mg/mL). UV-Vis spectrophotometer was used to measure the mixed solution at 517 nm after 15 minutes of incubation at room temperature. Methanol (0.75 mL) mixed with methanolic DPPH (1.5 mL) was used as blank. Ascorbic acid was used as standard chemical for calibration

curve construction. The free radical scavenging activity was reported as IC_{50} (antioxidant quantity required for 50% reduction of DPPH concentration) (0-25 mg/L).

STATISTICAL ANALYSIS

The assays were performed in triplicate and the results were expressed as average values and standard deviations (SD). The significant differences represented by letters were obtained by a one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$). Correlations were established using Pearsons correlation coefficient (r) inbivariate linear correlations ($p < 0.05$). These correlations were calculated using Microsoft office Excel 2010 (Microsoft Corporation, New Mexico, U.S.A) and SPSS version 20.0 (IBM corporation, New York, U.S.A).

RESULTS AND DISCUSSION

PHYSICAL ANALYSIS

The data in Table 2 showed the results of pH, moisture content, fat, diatery fibre, ash, total soluble solid, conductivity and acidity. From 11 samples examined, all samples are within the range of Malaysian standard for Kelulut. The pH range (3.22-3.69) ($p < 0.05$) as shown in Table 2 were similar with the studies conducted by Chuttong et al. (2016) in Thailand and do Nascimento et al. (2015) in Brazil. Low pH value may forbid the growth of microorganisms and thus prevent the contamination of honey (do Nascimento et al., 2015).

The moisture content is important in honey for determining its shelf-life (Lage et al. 2012; Shahnawaz et al. 2013). High moisture content may lead to fermentation of honey and loosing of the flavor and then the honey will lost its quality. The results showed moisture content ranging from 22.1% to 34.6% and were significantly different. This can be concluded that the moisture content has exceeded the limits specified by international standard regulation which is less than 20% for *Apis mellifera sp*. The results are in agreement with the studies by Chuttong et al. (2016). Moisture content may be high due to the humid environment and it become the most relevant characteristics of honey as it is a factor that influences viscosity, specific weight, maturation, crystallization and taste of the honey (do Nascimento et al. 2015). In conclusion, higher moisture content will lead to lower shelf-life.

Ash content in honey may represent minerals in honey, although in a very small amount present. This could be the indicator of the botanical and geographical origin of the honey. This study have small content of ashes for all samples. In comparison, ash content in other studies were higher. Similar values were obtained by de Almeida-Muradian et al. (2013). Our results of ash content shown in Table 3.1 were complied with the Malaysian standard which is less than 1%. Since they were less than 1%, all samples were not significantly difference ($p > 0.05$) from each other with only small amount of ash present.

Total soluble solids or Brixº is measured by refractometer. Soluble solids in honey are substances which dissolved in water such as sugars, acids, proteins, phenols, salts, and organic molecules (Colucci et al. 2016). According to Guerrini et al. (2009), Brixº could be used to determine honey adulteration because it is related to the sugar levels in honey. In present study, the total soluble solids (Table 2) of Johor stingless bee honey ranged from 68.70 ± 0.40 to 81.8 ± 0.50 Brixº ($p < 0.05$).

According to Acquarone et al. (2007), electrical conductivity is closely related to the ash and mineral contents in honey. For this parameter, it had been used to determine the botanical and also geographical origin of honey. International honey commission (2002) had denominated the maximum value of electrical conductivity of honey from *Apis mellifera sp.* of not more than 0.8 mS/cm for bloosom honey and more than 0.8 mS/cm for honeydew honey. According to the results for all samples tested, the values of electrical conductivity of samples were statistically difference and below 0.8 mS/cm.

Nweze et al. (2017) reported a significant difference of electrical conductivity from *Hypotrigona sp*. honey compared to *Apis mellifera sp.* and *Melipona sp*. honey, while both *Apis mellifera sp*. and *Melipona sp*. showed no significant difference. Similarly, de Almeida-Muradian et al. (2013) had compared between both *Apis mellifera sp*. and *Melipona sp*. (*Melipona subnitida*) whereby in their study, the electrical conductivity for both study did not exceed the limit (284.00 ± 5.00 and 102.77 ± 1.31 µS/cm). However, in Thailand, a study on 28 honey samples of *Trigona sp*. by Chuttong et al. (2016) exceeded the limit with the average of 1.1 ± 0.780 mS/cm. Among 28 samples of Thailand *Trigona sp.* honey samples, four samples showed mean values more than 2 mS/cm. In comparison to previous studies, our data showed similar results with Thailand *Trigona sp*. honey samples.

Acidity of the samples were ranging from 44.2-195.8 meq/kg ($p < 0.05$) (Table 2). International honey standard (2002) recommended the maximum of 50 meq/kg of acidity of honey from *Apis mellifera sp.* However almost all samples significantly exceeding the limit value. With comparison to other studies, most stingless bee honey have high acidity (Chuttong et al. 2016; Lage et al. 2012). This could be the outcome of fermentation process. Chuttong et al. (2016) showed a great high acidity in three species of *Melipona* honey (440-592 meq/kg). do Nascimento et al. (2015), however, have all samples following the limit value of acidity. de Almeida-Muradian et al. (2013) in their study also following the standard regulation for acidity.

The data of protein and carbohydrate were shown in Table 3. The protein content of honey dependent on the botanical and geographical origins. According to de Almeida-Muradian et al (2013), protein content in honey, play important role for honey formation, may determine the freshness of the honey in overheated, adulterated, and long honey storage. Current results showed that the protein content of Johor stingless bee honey was significantly different with a range from 0.58 ±

TABLE 2. Average values of the physicochemical properties of stingless bee honeys at various regions in Johor

Samples	Physicochemical parameters							
	pH	Moi (% w/w)	Fat (% w/w) (% w/w)	Dietary Fibre (% w/w)	Ash (% w/w)	TSS (ºBrix)	Cond. ($mScm^{-1}$)	Acidity ($meqkg^{-1}$)
TLD	3.39 ± 0.01^{bcd}	34.5 ± 0.12^{e}	0	0	0.03 ± 0.015^{ab}	69.00 ± 0.66^{a}	0.4540 ± 0.0035^{c}	85.8 ± 2.89^{b}
TKL	3.22 ± 0.03^{a}	33.3 ± 0.27^{e}	0	0	0.03 ± 0.010^{ab}	69.73 ± 0.32^{ab}	0.5857 ± 0.0035^{e}	195.8 ± 2.31^{f}
TMS	3.26 ± 0.03^{a}	33.6 ± 0.21^{e}	0	0	0.02 ± 0.012^{ab}	68.70 ± 0.40^{a}	0.5350 ± 0.0010^{d}	151.7 ± 5.00^{d}
TKU	3.29 ± 0.01^{ab}	29.9 ± 0.12^{b}	0	0	0.03 ± 0.012^{ab}	74.07 ± 0.15^{e}	0.7603 ± 0.0081^{h}	119.2 ± 3.82^{c}
TIBD	3.37 ± 0.07^{bc}	34.4 ± 0.98^{e}	0	0	0.01 ± 0.006^{a}	69.60 ± 0.27^{ab}	0.7127 ± 0.0040^{g}	131.7 ± 1.44^{cd}
TJB	3.57 ± 0.01^{e}	29.2 ± 0.63^{b}	0	0	0.04 ± 0.006^{ab}	73.07 ± 1.08^{e}	0.7030 ± 0.0236^{g}	144.2 ± 6.29^{d}
TMU	3.39 ± 0.01^{cd}	32.9 ± 0.84^{e}	0	0	0.04 ± 0.012^{ab}	70.93 ± 0.58^{b}	0.6620 ± 0.0061^{f}	162.5 ± 2.89^{e}
TP	3.48 ± 0.01^{de}	34.2 ± 0.75^{e}	0	0	0.07 ± 0.030^{b}	69.50 ± 0.36^{ab}	0.5420 ± 0.0096^{d}	112.5 ± 5.20^{e}
TBP	3.43 ± 0.01^{cd}	29.4 ± 0.78^{b}	0	0	0.05 ± 0.031^{ab}	74.03 ± 1.01^{e}	0.1941 ± 0.0003^{a}	65.8 ± 3.82^{b}
TSG	3.37 ± 0.08^{bc}	34.6 ± 0.10^{e}	0	0	0.02 ± 0.010^{ab}	70.27 ± 0.15^{ab}	0.5770 ± 0.0035^{e}	192.5 ± 4.33^{g}
TKT	3.69 ± 0.02^{f}	22.1 ± 0.50^{a}	0	0	0.04 ± 0.021^{ab}	81.8 ± 0.50^{d}	0.2433 ± 0.0055^{b}	44.2 ± 5.77^{a}

T: *Trigona spp.*, LD: Ledang, KL: Kluang, MS: Mersing, KU: Kulai, IBD: Institute Bioproduct Development, JB: Johor Bahru, MU: Muar, P: Pontian, BP: Batu Pahat, SG: Segamat, KT: Kota Tinggi, Moi: moisture, TSS: total soluble solids, Cond.: Conductivity. Data are expressed as the mean ± standard deviation (SD) (n = 1). Mean values are significantly different ($p < 0.05$) except for ash ($p > 0.05$). Mean values in each column with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

TABLE 4. Correlation matrix between physical, biochemical and antioxidant activity of 11 samples of Johor stingless bee honey

	pH	Moi	Ash	Cond.	TSS	HMF	Acidity	Protein	Carbohydrate	TPC	TFC	DPPH:IC_{50}
pH	1.000											
Moi	**-0.681**	1.000										
Ash	0.194	-0.088	1.000									
E.C	**-0.421**	**0.482**	-0.205	1.000								
TSS	**0.694**	**-0.971**	0.092	**-0.480**	1.000							
HMF	0.138	-0.312	0.176	**-0.663**	0.283	1.000						
Acidity	**-0.555**	**0.601**	-0.133	**0.705**	**-0.585**	**-0.461**	1.000					
Protein	**0.590**	-0.427	0.086	0.075	0.314	-0.003	-0.090	1.000				
Carbohydrate	**0.503**	**-0.924**	0.057	-0.558	**0.940**	0.335	-0.623	0.050	1.000			
TPC	-0.316	0.136	**-0.353**	**0.831**	-0.155	**-0.625**	**0.550**	0.173	-0.217	1.000		
TFC	-0.338	0.070	-0.222	-0.070	-0.083	0.093	0.141	-0.452	0.113	0.014	1.000	
DPPH:IC_{50}	-0.226	**0.554**	-0.218	-0.003	**-0.552**	-0.218	-0.051	-0.223	-0.517	-0.235	0.011	1.000

Moi: Moisture, C: Conductivity, TSS: Total soluble solid, HMF: Hydroxymethylfurfural, TPC: Total phenolic content, TFC: Total flavonoid content,DPPH:1,1-Diphenyl-2-picrylhydrazyl,IC_{50}:Inhibition concentration minimized at 50%. Data are expressed as the correlation coefficient, r.

TABLE 3. Average values of the biochemical properties and antioxidant of stingless bee honeys at various regions in Johor

Samples	Physicochemical parameters					
	Protein (% w/w)	CHO (% w/w)	HMF	TPC (mg kg^{-1})	TFC (mg GA/100g)	DPPH/IC_{50} (mg/ml) (mg RE/100g)
TLD	1.03 ± 0.0073^{d}	64.41 ± 0.131^{a}	0.03 ± 0.006^{a}	576.57 ± 2.695^{e}	46.57 ± 3.331^{b}	29.56 ± 0.91^{c}
TKL	1.19 ± 0.0069^{f}	65.48 ± 0.269^{a}	0.15 ± 0.101^{ab}	778.23 ± 2.011^{f}	91.30 ± 1.151^{d}	23.87 ± 0.48^{a}
TMS	0.82 ± 0.0003^{b}	65.58 ± 0.212^{a}	0.47 ± 0.367^{bc}	607.97 ± 4.605^{d}	194.98 ± 0.350^{g}	26.83 ± 0.90^{b}
TKU	0.61 ± 0.0058^{a}	69.43 ± 0.130^{b}	0.20 ± 0.058^{abc}	773.37 ± 1.514^{f}	76.09 ± 0.766^{c}	23.38 ± 0.34^{a}
TIBD	0.79 ± 0.0339^{b}	64.74 ± 0.904^{a}	0.06 ± 0.012^{a}	738.16 ± 7.354^{e}	136.16 ± 1.990^{f}	26.67 ± 0.06^{b}
TJB	5.68 ± 0.0034^{i}	65.09 ± 0.610^{a}	0.49 ± 0.017^{bc}	745.47 ± 3.431^{e}	36.67 ± 1.151^{a}	24.35 ± 0.51^{a}
TMU	0.90 ± 0.0056^{c}	66.13 ± 0.83^{a}	0.04 ± 0.058^{a}	747.91 ± 8.018^{e}	134.69 ± 0.752^{f}	23.38 ± 0.31^{a}
TP	1.32 ± 0.0405^{g}	64.46 ± 0.830^{a}	0.02 ± 0.006^{a}	530.17 ± 8.381^{b}	46.36 ± 3.762^{b}	24.86 ± 0.53^{a}
TBP	1.10 ± 0.0107^{e}	69.37 ± 0.923^{b}	5.02 ± 0.181^{d}	414.53 ± 3.166^{a}	106.84 ± 3.010^{e}	24.17 ± 0.19^{a}
TSG	0.58 ± 0.0001^{a}	64.79 ± 0.061^{a}	0.05 ± 0.010^{a}	587.60 ± 9.541^{c}	95.06 ± 3.041^{d}	26.43 ± 0.44^{b}
TKT	2.24 ± 0.0003^{h}	75.59 ± 0.511^{c}	48.68 ± 0.087^{c}	573.93 ± 3.729^{e}	106.94 ± 2.542^{e}	23.37 ± 0.36^{a}

T: *Trigona spp.*, LD: Ledang, KL: Kluang, MS: Mersing, KU: Kulai, IBD: Institute Bioproduct Development, JB: Johor Bahru, MU: Muar, P: Pontian, BP: Batu Pahat, SG: Segamat, KT: Kota Tinggi, CHO: Carbohydrate, TPC: Total pheolic content, TFC: Total flavonoid content, IC_{50}: Inhibition concentration minimized at 50%. Data are expressed as the mean ± standard deviation (SD) (n = 1). Mean values are significantly different ($p < 0.05$). Mean values in each column with the same letter are not significantly different ($p < 0.05$) by the Tukey's test.

0.0001% to 5.68 ± 0.0034% which belong to TSG and TJB stingless bee honey. Protein content in *Melipona subnitida* was lower than the minimum protein content of the present samples (0.28 ± 0.01%) (de Almeida-Muradian et al. 2013). Although protein content in stingless bee honey is not included in the Malaysian standard for stingless bee honey, however it is required for labelling of the stingless bee honey for commercialization.

Carbohydrate content in all Johor stingless bee honey samples is ranged from 64.41 ± 0.131% to 75.59 ± 0.511% ($p < 0.05$). According to Chua and Adnan (2014), presence of dietary fibre is an indication of unavailable carbohydrates. Since the present data showed no presence of dietary fibre (Table 2), then the carbohydrate measured in 11 samples of Johor stingless bee honey are in the category of available carbohydrate. In addition, there was no presence of fat in all samples as shown in Table 2.

BIOCHEMICAL ANALYSIS

The HMF content in ten samples were following Malaysian Standard (2017) for tropical regions which less than 30 mg/kg. All honey samples except one sample (TKT) contained very low amount of HMF in honey. TKT showed high HMF content compared to other samples which is 48.68 mg/kg, more than the limit set by Malaysian Standard (2017) but lesser than 80 mg/kg as in international honey regulation (International Honey Commission, 2002) (Table 3). All samples were significantly different. Hydroxymethylfurfural content is a recognizable parameter to determine the freshness of honey. Increasing of HMF content may be due to several factors such as time and temperature of heating, storage conditions, pH and floral sources (de Almeida-Muradian et al. 2013). HMF often occur due to the acid-catalyzed dehydration of hexose (fructose and glucose) or from Maillard's reaction (Al-Diab and Bushra Jarkas 2015).

In present study, total phenolic content of eleven Johor stingless bee honey is ranged from 414.53 ± 3.166 to 778.23 ± 2.011 mg GAE/100g ($p < 0.05$) as shown in Table 3. Total phenolic content presented the polyphenol compounds in honey. Polyphenol compounds are important compounds that contribute to the appearance and functional properties of honey (Bakar et al. 2017). A study by Bakar et al. (2017) measure the total phenolic content of stingless bee honey from two states in Malaysia, Melaka and Johor with two different solvent, water and methanol. In both solvents, the total phenolic content was ranged from 357.14 ± 3.57 to 520.83 ± 4.49 mg GAE/kg. This results is lower compared to the present data of Johor stingless bee honey.

The present result contained higher total phenolic content when compared to *Trigona* honey from South Sulawesi, Indonesia with average of 106.0 mg/100 g (Syam et al. 2016). Another study from Kek et al. (2014) showed a comparison of total phenolic compound in two species of bees, *Apis mellifera sp*. and *Trigona sp*. whereby *Trigona sp*. have higher phenolic content than *Apis mellifera sp*. Three species of stingless bee honey from Sarawak, Malaysia was experimented and the total phenolic content for all samples was ranged from 44.72 ± 6.50 to 99.04 ± 5.14 mg/ml (Tuksitha et al., 2018).

Total flavonoid content of honey was measured using the aluminium chloride method (Rahaman 2014) and the results was shown in Table 3. Total flavonoid content in honey is responsible for the aroma and antioxidant potentiality. Flavonoid content in honey is dependent on the floral origin of honey, including bee species and foraging area of the bees (Saxena et al. 2010; Olievera et al. 2017). The flavonoid content in Johor stingless bee honey was significantly difference ($p < 0.05$) and ranged from 36.67 ± 1.151 to 194.98 ± 0.350 mg RE/100g. The data from present study is similar to the data from Olievera et al. (2017), although with different type of stingless bees (*Melipona sp.*) which ranged from 30.24 ± 2 to 279.73 ± 4.6 mg QE/g. Oddo et al. (2008) measured flavonoid content in Australian stingless bee (*Trigona carbonara sp*.) and the flavonoid content was significantly lower than the current study (10.02 ± 1.59 mg QE/100 g). Tuksitha et al. (2018) reported significant differenced of total flavonoid content from three species of Sarawak stingless bee honey (12.41 ± 0.62 to 17.67 ± 0.75 mg/ml). Flavonoid content in a study reported by Bakar et al. (2017) showed a significant variability in all samples (both distilled water and methanol) with a range between 53.81 ± 4.12 to 549.05 ± 9.74 mg rutin/kg. Both phenolic and flavonoid content are strong antioxidant content in honey which have a potential in scavenging free radicals causing the radical species in more stable and less reactive condition (Bakar et al. 2017).

ANTIOXIDANT ANALYSIS

2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis is used to evaluate the scavenging ability of tested substances. In this analysis, a purple solution of DPPH reagent reacts with given substances and changes color to light yellow to colorless. This may be due to the scavenging activity of compounds insubstances to the DPPH reagent (Tuksitha et al. 2018). There are several factors involved in antioxidant potential in substances which are botanical origins, climates, surrounding of hives, harvesting method, and pigments of the honey (Bakar et al. 2017). The data is expressed as IC_{50} that represents the amount of antioxidant needed to minimize the initial concentration of DPPH by 50%. The present result (Table 3) ranged from 23.37 ± 0.36 to 29.56 ± 0.91 mg/ml which is in agreement with the DPPH result from Tuksitha et al. (2017) (17.0 ± 7.5 to 47.4 ± 3.2 %) and were significantly difference ($p < 0.05$).

CORRELATION BETWEEN PARAMETERS

Table 4 showed the correlation between physicochemical parameters for Johor stingless bee honeys. pH showed a positive correlation with total soluble solids ($r = 0.694$) and negative correlation with moisture ($r = -0.681$), electrical conductivity ($r = -0.421$), and acidity ($r = -0.555$). A strong

correlation was obtained between moisture content and total soluble solids which can be explained that the lower content of soluble solids will lead to high moisture in honey (Moo-Huchin et al. 2015).

A strong correlation was also obtained between acidity and moisture (r = 0.601) and electrical conductivity (r = 0.705) but moderately negative correlation with soluble solids (r = -0.585) and HMF (r = -0.461). A study by Fallico et al. (2004) showed a relationship between acidity and HMF and pH. Khalil et al. (2010) showed a strong correlation between HMF and total acidity (r = 0.763). Protein showed moderate correlation with pH (r = 0.590) and carbohydrate showed moderate correlation with pH (r = 0.503), negatively high with moisture content (r = -0.924) and a strong correlation with total soluble solid (r = 0.940). Antioxidant activity, however have a very low correlation with phenolic content (r = -0.235) and flavonoid content (r = 0.011). This indicates that polyphenol content may not be the only factors that increase the antioxidant activity in Johor stingless bee honeys (Ahmed et al. 2014).

CONCLUSION

This is the first report of Johor stingless bee honey on the physicochemical properties. This data can contribute to useful knowledge for future study regarding the correlation between physicochemical and nutritional composition of the Malaysian stingless bee honey from different botanical origins.

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