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Physicochemical characteristics, antioxidant content and antimicrobial activity of stingless bee honey

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It has been commonly recognised that honey produced by stingless bee or locally known as *Kelulut* honey possessed various health benefits. However, scientific study on its physicochemical, antioxidant and antimicrobial properties are very limited. The present study aimed to determine the physicochemical properties, antioxidant content and antimicrobial activity of stingless bee honey (SBH). Four different colour of SBH were collected from Agropolis, UniSZA and designated as honey samples A, B, C and D. Physicochemical analysis of SBH included colour, pH, moisture, ash, and total soluble solids. Antioxidant activity analysis included total phenolic, total flavonoid and ascorbic acid equivalent antioxidant activity (AEAC). For antimicrobial activity, it was determined by using agar diffusion assay against *Escherichia coli, Staphylococcus aureus, Salmonella* Typhimurium, and *Listeria monocytogenes*. The SBH have 31.63 to 35.40% moisture content, 0.11 to 0.41% ash content, total soluble solids ranged from 69.80 to 72.63 °βrix and pH of 2.95 to 4.10. Colour measurement showed SBH sample A was darkest (L* = 22.65). SBH sample B showed the highest content of total phenolic and flavonoid which are 4.70 mg Gallic acid equivalents (GAE)/g and 2.39 mg quercetin equivalents (QE)/g respectively. In term of antioxidant content, SBH sample D had the highest AEAC value which is 3.20 mg AEAC/g. For antimicrobial activity, all SBH samples showed antimicrobial activity against *E.coli, S. aureus, Salmonella* Typhimurium and *Listeria monocytogenes* where honey sample B showed the highest antimicrobial activity against all bacteria tested except for *S. aureus*.

Keywords: Stingless bee honey, physicochemical, antimicrobial

INTRODUCTION

Honey is sweet and viscous liquid well known for its health benefits and has many application in food. It can be used to prepare simple drink or as an ingredient to variety of food products. This sweet, waxy and viscous liquid is produced by honey bees after consuming floral nectar. Stingless bee or meliponines can be found mainly in the tropical and subtropical regions, which include Central and South America, Australia, Indonesia, Thailand and Malaysia. Stingless bee have more than 500 species from approximately 60 genera compared to widely known honey bee consist of 11 species from genus *Apis* (Kek et al., 2018).

The physicochemical properties and functional properties of honey are affected by the source of nectar consumed by the bee and the processes involved (Chan et al., 2017; Kek et al., 2018). Honey processing equipment also plays an important role in determining the processed honey physicochemical characteristics such as color, moisture and viscosity. These parameters will determine the quality of honey which affect its price, stability and shelf life (Kek et al., 2018). Health benefits of honey mainly due to its antioxidant content that is capable to scavenge radicals and reduce oxidative stress. Studies

have shown that the source of floral nectar collected by honey bee significantly affect the amount of bioactive compound such as phenolic acids, flavonoids and carotenoids present in the honey (Chan et al., 2017). Thus, honey can varied greatly in the antioxidant content and physicochemical characteristics.

In term of antimicrobial activity, the level of its antimicrobial potency is directly related to its physicochemical characteristics and the target microbes. Geographical area, climate, season, and hive, foraging bees and floral sources have significant effect on honey antimicrobial activity (Zainol, 2016). It however can be influenced by human activities during processing such as harvesting methods, storage, packaging, transportation and physical contact (Zainol, 2016).

The stingless bee (*Lebah Kelulut*) produce only a small amount of honey yield per hive basis compared to *Apis mellifera*. The limited yield of stingless bee honey (SBH) resulted little study being carried out on its physicochemical and functional properties. SBH has been found to have different colour and it indicates the presence of pigments which might have bioactive compounds such as phenolics and flavonoid. Therefore, the objectives of this study were to determine the physicochemical characteristics, antioxidant content and antimicrobial activity of SBH.

MATERIALS AND METHODS

Samples

In this study, the sample used was SBH collected from Apiary Farm, Agropolis, UniSZA Kampus Besut. The SBH were labeled based on the colour of the SBH and designated as SBH A, B, C and D (Figure 1). SBH A and B were collected 12 to 18 months and kept at room temperature before analysed. On the other hand, SBH sample C and D were freshly harvested and stored at chilling temperature (5 to 7 °C).

Physicochemical analysis

pH

Approximately 2 g of sample diluted with 15mL distilled water were measured using pH meter (Thermo Scientific Orion 2-Star Benchtop) (Roslan, 2017).

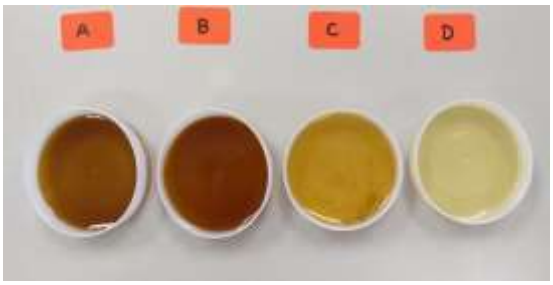

Figure 1; Visual observation the colour of different sample of stingless bee honey

Moisture content

The crucible was oven dried at 105 °C for 4 h and was let to cool in the dessicator. The cooled oven dried crucible was weighed as W1. Next, about 3 g of SBH sample was added into the crucible and weighed as W2. After that, the crucible containing SBH was heated in oven at 105 °C for 24 h. After 24 h, the crucible containing SBH was cooled down in a dessicator and weighed as W3. Finally, percentage of moisture content was calculated by using formula (Roslan, 2017):

$$\%\, moisture\ = \frac{W2-W3}{W2-W1} \times 100$$

Where;
W1 = Weigh of crucible with in grams
W2 = Weigh of sample with crucible before drying in grams
W3 = Weigh of sample with crucible after drying in grams

Ash content

The crucible was oven dried for 1 h at 105 °C. It was cooled in a dessicator and weighed as W1. Then, about 3 g of SBH was weighed (W2) and the sample was ignited in muffle furnace at temperature 550 °C. The ash of SBH was weighed and recorded as W3. The ash content was calculated using the following formula**:**

$$\%\, ash = \frac{W3 - W1}{W2}\ X\ 100$$

Where:
W1 = weight of crucible (g)
W2 = weight of crucible + sample (g)
W3 = weight of crucible + ash (g)

Colour

SBH colour was measured by Chromameter (CR-400/410 Konica Minolta) device. The device was first calibrated before use by using white calibration plate and an illuminant. Then, the

button was pressed to analyse the sample and the results were showed in term of colour parameters which are L*, a* and b* where L* represented lightness of the sample, a* represented change in hue from red to green and b* represented change in hue from yellow to blue (Yusri Azhar, 2012).

Total soluble solids

The total soluble solid of SBH was determined using refractometer (Atago). SBH was spread on the prism of the refractometer and the readings were measured as °Brix.

Antioxidant analysis

Total phenolic content

The total phenolic content was determined by using the Folin-Ciocalteu assay (Chan et al., 2017). First, 0.5 mL of 10% SBH solution was mixed with 2.5 mL of 0.2N Folin-Ciocalteu reagent for 5 min. After that, 2 mL of 7.5% Na_2CO_3 solution was added and incubated at room temperature for 2 h. Then, the total phenolic content was measured at 760 nm by using spectrophotometer (UV-1240 Shimadzu) against methanol as blank. Next, a standard curve was created (R^2 = 0.987) using Gallic acid (50, 100, 150, 200, 250, 300, 400 mgL^{-1}) and the total phenolic content of SBH was expressed in mg Gallic acid equivalents (GAE)g^{-1} honey.

Total flavonoid content

The total flavonoid content in SBH was determined using method as described by Moniruzzaman et al., (2013). First, 1 mL of honey extract was mixed with 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$ (wv^{-1}). After 5 min, 0.3 mL of 10 % AlCl3 (w/v) was added before the addition of 2 mL NaOH (1M) after 6 min. Next, 2.4 mL distilled water was added and the mixture was mixed vigorously. The total flavonoid content was measured using spectrophotometer (UV-1240 Shimadzu) with wavelength set at 415 nm against a blank sample consisting of a 1 mL SBH with 1 mL methanol. Standard curve was created using a standard solution of quercetin (25, 50, 75, 100, 150, 200 mgL^{-1}) and express the results as mg quercetin equivalents (QE)g^{-1} of honey.

Antioxidant content

Antioxidant content in SBH was determined using the method described by (Moniruzzaman et al., 2013). First, SBH was dissolved in methanol until it reach the final concentration of 0.03 gmL^{-1}. Next, 1.50 mL of the methanolic SBH solution was mixed with 3 mL of a 0.02 $mgmL^{-1}$ DPPH solution. After 15 min, the mixture was measured at 517 nm using Spectrophotometer (UV-1240 Shimadzu) against the blank containing 1.50 mL of a methanolic SBH solution with 3 mL methanol. The standard solution for calibration curve is ascorbic acid (60, 120, 180, 240, 300, 360, 420, 480 μgmL^{-1}) prepared in distilled water and the result is express as mg of ascorbic acid equivalent antioxidant activity (AEAC) per g honey.

Antimicrobial activity

Preparation of bacteria culture

One loop of *Escherichia coli* (ATCC 35219) was taken from slanted nutrient agar and then being added into tryptic soy broth (TSB). Then, it was incubated at 37 °C for 24 h in shaker incubator at 150 rpm. The same procedure was repeated for *Staphylococcus aureus, Salmonella* Typhimurium (ATCC 14028) and *Listeria monocytogenes* (ATCC 3932).

Disc diffusion method

The overnight culture was used to prepare bacteria culture with turbidity of 0.5 McFarland. Then, sterile cotton swab was used to spread the bacteria onto Mueller Hinton agar plate until it form a uniform lawn. Any excess moisture on the agar surface was allowed to be absorbed prior to applying the empty discs. Next, 20 µL of SBH solution which are SBH A, B, C and D were added onto the disc. The plates were incubated at 37 °C for 24 h and the zone of inhibition diameter was measured after 24 h.

Statistical analysis

All of the analysis were performed in triplicates. The data obtained were reported as mean ± standard deviation. One-way ANOVA was used to determine the statistical significance of the result at significant level $P < 0.05$ by using SPSS software.

RESULTS AND DISCUSSION

Physicochemical analysis

The moisture, ash, total soluble solids, and pH of the SBH is shown in Table 1. Physicochemical composition of SBH are important parameters which determine the quality of SBH.

Table 1; Physicochemical properties of stingless bee honey (SBH)

Parameters	SBH			
	A	B	C	D
Moisture (%)	33.30^{b}±0.16	35.18^{c} ±0.21	35.40^{c} ±0.27	31.63^{a} ±0.41
Ash (%)	0.41^{d} ±0.02	0.33^{c} ±0.02	0.19^{c} ± 0.02	0.11^{a} ± 0.02
Total soluble solid (°βrix)	71.27^{b} ±0.25	72.63^{c} ±0.57	69.80^{a} ±0.53	70.07^{a} ±0.31
pH	2.95^{a} ±0.11	3.09^{a} ± 0.01	4.10^{b} ± 0.02	3.88^{b} ± 0.21

The different letters (superscript) within the rows indicate statistically significant differences ($p < 0.05$).

Moisture content

The amount of moisture in honey will affect the freshness and preventing yeast fermentation which will lead to spoilage (Biluca et al., 2016). The results in Table 1 shows the percentage of moisture of SBH honey were ranged 31.18 to 35.40%. The values were significantly higher than the 20% maximum allowed by international standards for *Apis mellifera* honey (Codex Alimentarius, 2001). All the SBH from this study showed a high moisture content. Malaysia hot and humid climate might be the contributing factor of the high moisture content in SBH as highlighted by Mustafa et al., (2018).

Ash content

Table 1 shows the percentage of ash content of SBH honey were about 0.11 to 0.41%. These values were within 0.03% to 1.23% as reported in previous studies (Al et al., 2009; Silva et al., 2009). The previous study on Tualang and Gelam honey has characterized that darker honey has higher mineral content than the light honey (Khalil et al., 2011). As ash content indicate the amount of mineral in honey, SBH A (0.44%) was found have the highest mineral content because it has the highest percentage of ash content and the colour was the darkest compared to SBH C and D according to L* value of colour analysis. The amount of mineral present in the honey was shown to be affected by the bee during the foraging on flora (Finola et al., 2007; Ojeda De Rodriguez et al., 2004).

pH

SBH C has the highest pH value which is 4.10 while SBH A has the lowest pH value at 2.95. Several factors may affect the pH such as extraction and storage conditions. The pH of honey will directly affect the honey texture, stability and shelf-life (Silva et al., 2009). The SBH A and B have low pH value compared to SBH C and D and this may due to their storage condition. SBH A and B were kept at room temperature for 12 to 18 month before being analysed. This has been proved by the previous study done by Chuttong et al. (2016) where the authors have reported that the total acidity of SBH increase when stored for 6 to 12 mth at room temperature.

Total soluble solids

Total soluble solids value of SBH honey were within 69.80 to 72.63 ºβrix. The findings in this study were similar with the study by Nordin et al. (2018), that reported the total soluble solids were ranged from 64.50 to 75.80 ºβrix in stingless bee honey. The soluble solids found in honey consists of sugars, organic acids, and minerals (Biluca et al., 2016). According to the grading system of the United States Department of Agriculture (USDA) (1985), high grade honey (A and B) has with total soluble solids greater than 81.4% and grade C honey will have total soluble solids between 80 to 81.3%. Honey with soluble solids less than 80% is likely to ferment during storage. Thus, the SBH tested in this study can be considered lack of stability during storage which may undergo fermentation.

Colour

Colour is the physical properties observed immediately by customers. Honey can be vary in colour considerable from light yellowish to black charcoal.The colour is greatly influence by the mineral content, nectar, pigment, pollen and phenolic content of the honey as well as its geographical origin (Solayman et al., 2016; Bertoncelj et al., 2007). Table 2 shows the value of L*, a* and b* of SBH. SBH D had the highest value of L* which is 34.62 whereas SBH A had the lowest value of L* which is 22.65. SBH A had the highest a* value which is 4.54 while SBH D had the lowest value of a* which is negative value (-1.84). The negative value of a* indicate that a green compound may present in the stingless bee honey sample A (Keng et al., 2017). The results in Table 2 shows the SBH C had the highest value of b* which is 17.96 and SBH D showed the lowest value of b* which is 12.27.

Table 2; Value of L*, a* and b* of stingless bee honey (SBH)

Colour	SBH			
	A	B	C	D
L*	22.65^{a}±1.40	24.61^{a}±0.63	29.38^{b} ±0.96	34.62^{c}±0.97
a*	4.54^{d}± 0.25	3.99^{c}± 0.06	2.79^{b} ± 0.39	–1.84^{a}±0.02
b*	14.95^{b}±0.76	17.11^{c}±0.18	17.96^{c} ±1.13	12.27^{a}±0.50

The different letters (superscript) within the rows indicate statistically significant differences ($p < 0.05$)

Figure 1 showed the visual observation of different colour of stingless bee honey samples. Among four SBH samples (A, B, C, and D), SBH D had the lightest colour whereas SBH A had the darkest colour. SBH C and D have the lighter colour than sample A and B were thought to be related to its floral nectar source as the area of the stingless bee honey was surrounded by pokok Gelam (*Melaleuca leucadendra*), "jambu laut" (*Syzgium jambos*) and pokok rumput (*Asystasia gangetica*). Previous study found honey produced by bees feeding on Gelam (*Melaleuca leucadendra*) will produce honey with colour of light amber (Praciak, 2013). Meanwhile, bees feeding on "jambu laut" (*Syzgium jambos*) will gives the nectar colour of amber (Morton, 2001). In addition, other factors such as fruit or flower season, bee species and maturity of the honey will also affect the colour (Ij et al., 2017).

Antioxidant analysis

Table 3; Antioxidant analysis of stingless bee honey (SBH)

Parameter	SBH			
	A	B	C	D
Total phenolic content (mg GAE/g)	3.57^{c} ± 0.34	4.70^{d} ± 0.34	2.12^{b} ±0.15	1.06^{a} ± 0.11
Total flavonoid content (mg QE/g)	1.04^{b} ± 0.16	2.39^{c} ± 0.15	0.16^{a} ± 0.01	0.22^{a} ± 0.03
AEAC (mg AEAC/g)	0.46^{a} ± 0.01	0.69^{b} ± 0.04	1.72^{c} ± 0.04	3.20^{d} ± 0.10

The different letters (superscript) within the rows indicate statistically significant differences ($p < 0.05$).

Total phenolic content

The total phenolic content of SBH was determined based on the colour reaction using Folin Ciocalteu reagent and the result of TPC is expressed in gallic acid equivalent (GAE) since gallic acid easily soluble in Folin Ciocalteu reagent (Amanarth, 2004). This method is simple and fast to measure the total phenolic content in complex matrix like honey (Chua et al., 2013).

Table 3 shows SBH B had the highest total phenolic content which is 4.70 mg GAE/g while SBH D had the lowest total phenolic content which is 1.06 mg GAE/g. The total phenolic content of SBH in this study were found to be higher than previous study done by Chan et al. (2017) which only ranging from 0.53 to 1.17 mg GAE/g. Apart from that, the total phenolic content of the stingless bee honey from this study also higher when compared to the honey from *Apis* spp. like Tualang, Gelam and Pineapple which are only 0.59 mg GAE/g, 0.60 mg GAE/g and 0.51 mg GAE/g respectively (Peng et al., 2014). The total phenolic content obtained from this study were also found to be higher than in Manuka honey which is 0.53 mg GAE/g (Moniruzzaman et al., 2013).

In addition, Moniruzzaman et al., (2013) has reported that colour intensity is a reliable parameter to determine the presence of pigments such as carotenoids, phenolics and flavonoids. The authors also reported sourwood honey which had a darker colour was found to contain higher phenolic and flavonoid. However, in this research, colour intensity was not measured but it can be correlate with the L* value (lightness) where decrease in lightness results in high colour intensity (Smith, 2015). The results obtained show that honey sample A and B have a higher total phenolic content compared to other samples as they have lower value of L*.

Total flavonoid content

Table 3 shows SBH B had the highest total flavonoid content which is 2.39 mg QE/g whereas SBH C had the lowest total flavonoid content with the value 0.16 mg QE/g. Chua et al., (2013) reported that the total flavonoid content of honey from *Apis.sp* which are Tualang, Gelam and Acacia have the flavonoid content with value 0.19 mg QE/g, 0.33 mg QE/g and 0.31 mg QE/g, respectively which are lower than the data obtained from this research. The TFC of other

SBH also show low concentration ranging from 0.04 to 0.08 mg QE/g (Chan et al., 2017). For Manuka honey, the TFC is found to be 0.10 mg QE/g (Monirruzaman et al., 2013).

Flavonoids consist of groups of low molecular weight phenolic compounds which directly affect the taste and antioxidant activity of honey (Moniruzzaman et al., 2013). Flavonoids are important antioxidant as they stabilize the reactive oxygen species (Nijveldt et al., 2001). Therefore, honey containing higher flavonoid concentrations is desirable due to their purported antioxidant potential.For flavonoid content, it was different from phenolic as it has the ability to resist degradation during heat processing and storage temperature and duration. The flavonoid structure, presence of oxygen or oxidizing agents will influence the flavonoid content in sample (Makris & Rossiter, 2000). Thus, the results from this study showed SBH A and B have the higher amount of total flavonoid than SBH C and D.

Antioxidant content

Besides polyphenols, ascorbic acid and enzymes such as glucose oxidase and catalase also known to act as antioxidant (Alvarez-Suarez et al., 2010). Table 3 shows the AEAC value of the SBH ranged from 0.46 to 3.20 mg AEAC/g. According to Kek et al. (2018), among Malaysian raw honeys, SBH had the highest AEAC value of 0.27 mg AEAC/g followed by Tualang, Gelam and Borneo honey. However, Manuka honey has AEAC value was 4.21 mg AEAC/g (Lachman et al., 2010) which is higher than SBH in this study.

Studies have shown storage duration will influence the concentrations ascorbic acid and several other compounds (Wang et al., 2006**)**. As the result obtained, SBH D has the highest AEAC value while SBH A has the lowest AEAC value followed by SBH B which also consider to has lower AEAC value because SBH A and B were stored at room temperature for a long term which 12 to 18 mth.

Antimicrobial activity

Table 4 shows the diameter of inhibition zone of honey samples and suggested that all SBH A, B, C and D have antibacterial activity against tested bacteria, namely Escherichia coli, Staphylococcus aureus, Salmonella Typhimurium and Listeria monocytogenes. However, the results show there were no significant difference in inhibition zone diameter zone between different SBH samples. Nevertheless, it can be seen that SBH B on average has larger inhibition zone compared to SBH A, C and D against the tested bacteria except for Staphylococcus aureus.

Studies found darker honey and high phenolic content show higher antimicrobial activity (Alvarez-Suarez et al., 2010; Estevinho et al., 2008). Flavonoids and phenolic acids are known to be responsible for antibacterial activity (Crushnie & Lamb, 2005). The antibacterial activity was thought to be contribute by the benzene ring and polyphenol side-chain (Tuksitha et al., 2018). SBH B has dark color (Table 2) and has the highest amount of total phenolic and flavonoid content (Table 3) which might explain the reason SBH B has greater antibacterial effect.

Table 4. Antimicrobial activity of Stingless Bee Honey (SBH) against *Escherichia coli, Staphylococcus aureus, Salmonella* Typhimurium, *Listeria monocytogenes.*

Organism	Inhibition zone (mm)			
	SBH A	SBH B	SBH C	SBH D
***Escherichia coli* (ATCC 35219)**	$16.0^{a} \pm 2.8$	$19.5^{a} \pm 0.7$	$16.0^{a} \pm 1.4$	$15.0^{a} \pm 2.8$
Staphylococcus aureus	$30.5^{a} \pm 0.7$	$26.0^{a} \pm 1.4$	$27.0^{a} \pm 4.2$	$26.0^{a} \pm 1.4$
***Salmonella* Typhimurium (ATCC 14028)**	$19.0^{a} \pm 2.8$	$20.5^{a} \pm 0.7$	$17.0^{a} \pm 1.4$	$19.5^{a} \pm 2.1$
***Listeria monocytogenes* (ATCC 3932)**	$22.0^{a} \pm 0.28$	$23.0^{a} \pm 0.0$	$21.5^{a} \pm 2.1$	$21.0^{a} \pm 0.0$

The different letters (superscript) within the rows indicate statistically significant differences ($p < 0.05$).

CONCLUSION

The different colour of SBH and storage duration were found to affect the physicochemical properties, antioxidant and antimicrobial activity. SBH B which is dark color has the highest total phenolic and flavonoid content. All SBH samples exhibit significant antibacterial effect against *E. coli, S. aureus, S.* Typhimurium and *L. monocytogenes*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

NAMH performed the experiment, data analysis and wrote, AAG and JYHT designed experiments and reviewed the manuscript. All authors read and approved the final version.

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