Pertanika J. Sci. & Technol. 27 (S1): 15 - 28 (2019)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Phytochemical Compositions and Antioxidant Activities of Malaysian Stingless Bee Honey

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ABSTRACT

The current study investigates the phytochemical composition of Malaysian stingless bee honey (Kelulut honey-KH), which consists of total phenolic (TPC) and total flavonoid content (TFC), and antioxidant activity. The honey was collected from five different regions in Malaysia i.e. south, central, eastern, northern and east coast regions. TPC and TFC were quantified by using Folin-Ciocalteu and the aluminum chloride colorimetric techniques, respectively. The antioxidant activity was investigated using two methods: 1) 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay; 2) ferric reducing/antioxidant power assay (FRAP). The findings indicated that there were significant differences in phytochemical compositions and antioxidant activities of KH between different regions. This implies that geographical location, as well as cultivation and treatment processes, have significant effects on the KH quality.

Keywords: Kelulut honey, stingless bee honey, trigona spp, total phenolic content, total flavonoid content, antioxidant

ARTICLE INFO

Article history:
Received: 24 October 2018
Accepted: 15 February 2019
Published: 21 June 2019

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INTRODUCTION

Stingless bee honey, like many other types of honey is a sugary liquid which has a superb taste and odor (Chuttong et al., 2016a). It is produced by a stingless bee,

ISSN: 0128-7680
e-ISSN: 2231-8526

commonly known as "Kelulut" in Malaysia (Kek et al., 2014; Zainol et al., 2013). Stingless bee honey belongs to the order *Hymenoptera* under the family *Apidae* and sub-family tribe *Meliponini* (Chuttong et al., 2016b). Kelulut is naturally active all the time except during cold and cloudy weather. They are highly sociable, with one queen living together with thousands of workers (Chuttong et al., 2015). The bees normally inhabit in tropical and subtropical regions globally like Central and South America, Africa, Asia and northern Australia (Boorn et al., 2010). Shadan et al. (2018) reported that the selling price of kelulut honey (KH) could be as high as $100/kg, which was almost double compared to the honey made by the regular honey ($20–40/kg). This is mainly due to high contents of flavonoids and polyphenols.

In recent years, the kelulut industry has gained wide attention in Malaysia. Particularly, KH has been reported to be useful for medical and therapeutic purposes (Kek et al., 2014). KH has different phytochemical attributes as compared to regular honey bee in term of color, taste, viscosity, water and sugar contents (Biluca et al., 2014). Furthermore, KH has higher contents of flavonoids and polyphenols in contrast to honey produced by the Apis spp. (Biluca et al., 2016; Rodriguez-Malaver et al., 2013; Rodriguez-Malaver et al., 2009). Additionally, KH is more fluid in texture and undergoes slow crystallization. KH can be divided into various types according to the physical and chemical components. These components are linked to the physiology of making of the raw material, the territorial site of the floral source, the species of bee and the conditions of the ecosystem in which the bees live (Tuksitha et al., 2018). A previous study demonstrated that KH be made of mainly carbohydrates, water, amino acids, vitamins and minerals (Chuttong et al., 2016a). Another study reported that KH possesses distinctive and divergent phenolic and flavonoid composites that shown to have a vital function with regard to its antibacterial, anti-inflammatory and antioxidant activities of the Borneo (Sarawak) stingless bee honey (Tuksitha et al.,2018).

The topographical and botanical regions and the form of bees have essential function in forming the biological structure of honey plus the total antioxidant capacity (Erejuwa et al., 2012). A review by Nordin et al. (2018) reported that the phytochemical properties of honey varied significantly owing to the huge differences in bee species and geographic starting point. Recently, Kek et al. (2017) and Abu Bakar et al. (2017) analyzed the phytochemical of KH from various geographical and species from Malaysia. The authors demonstrated that KH had greater total phenolic content (TPC) with average amounts of 784.3 mg GAE/kg compared to the regular honey (Kek et al., 2014). The antioxidant activity of KH displayed superior antioxidant potential in comparison with another type of honey (Abu Bakar et al., 2017). Although, there have been few studies investigated on the phytochemical properties of KH from Malaysia, however, these studies only examined KH from peninsular Malaysia and some southern states. There are no studies

on the phytochemical and antioxidant properties of KH from other regions of Malaysia. Besides, there are no comparative studies on phytochemical properties and antioxidant activities of KH from different regions of Malaysia. Furthermore, because of limited data, there is a scarcity of characteristics and quality standards available for the KH. This is mainly due to variations in the standards established by the international honey standards (Codex Alimentarius Commission, 2001). Thus, this paper investigated the phytochemical properties and antioxidant activities of KH obtained from the southern, central, eastern, northern and east coast (Sarawak and Sabah) regions of Malaysia.

MATERIALS AND METHODS

Kelulut Honey Samples

One liter of KH samples produced by stingless bees from different regions i.e. south (Johore), central (Selangor), eastern (Kelantan), northern (Kedah) and east coast regions (Sabah and Sarawak) were purchased from respected local KH collectors between September to November 2017. It should be noted that all of the samples were cultivated by feeding with multifloral nectar source. The details of the samples region and time of collection are as tabulated in Table 1. All of the samples were kept in sterile airtight glass bottles at room temperature to prevent the absorption of moisture for the duration of sampling, storage and analytical test. In order to evaluate the pH values, color intensity, TPC, TFC and antioxidant capacity i.e. DPPH and FRAP activities, about two grams of KH were diluted with 20 mL of distilled water to produce 0.1 g/mL of concentration. Each analysis was repeated 3 times.

Table 1
KH samples region and time of samples collection

Sample Code	Time of collection	Region
Central	September 2017	Pusat Floral Cheras, Selangor
South	October 2017	Pusat Pertanian Parit Botak, Johore
East	October 2017	Kampung Rasal, Tok Uban, Pasir Mas, Kelantan
Sarawak	October 2017	Pusa District, Sarawak
North	November 2017	Pusat Pertanian Teluk Chengai, Kedah
Sabah	November 2017	Taman Pertanian Tenom, Sabah

Phytochemical Analysis

TPC and TFC of KH were analyzed by using Folin-Ciocalteu reagent (Berretta et al., 2005) and the aluminum chloride colorimetric technique (Ali, et al., 2015), respectively as shown in Figure 1. A total of 200 µL of KH solution was added to 3 mL of 10% diluted Folin-Ciocalteu reagent. Later 90 minutes of placing the solution in the dark

at room temperature, the absorbance was quantified using a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510) at 750 nm. The TPC value was stated as mg gallic acid equivalents per 100 g of honey.

For measuring TFC, 1 mL of the KH sample (0.1-0.4 g/mL) was mixed with 2% of aluminum chloride solution. Following the incubation for 10 minutes at 25°C, the absorbance of the mixture was quantified at 430 nm via a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510). TFC was expressed in mg QUE/ 100 g FW by using the calibration curve according to quercetin standard.

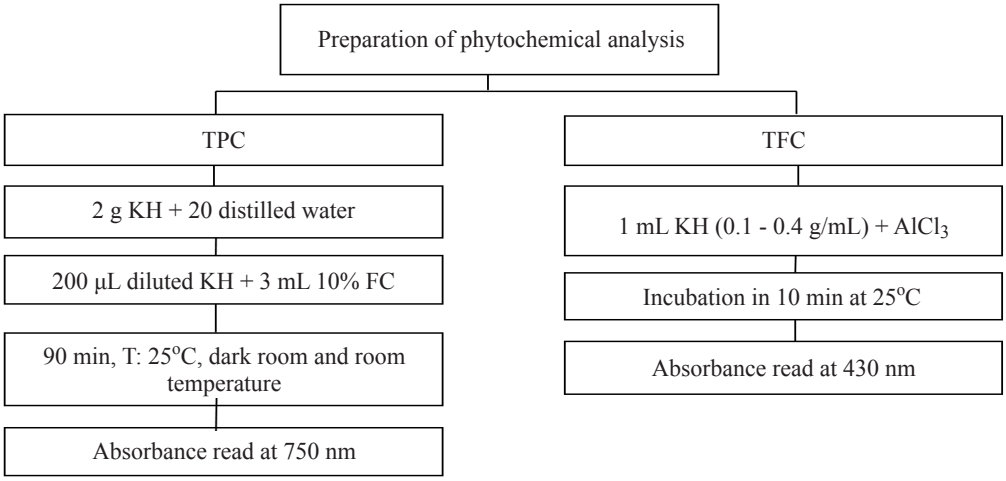

Figure 1. Flowchart representing steps in TPC and TFC determination

Antioxidant Activity

DPPH. The principle of DPPH method is based on the electron transfer, where it measures the scavenging activity of a particular sample (Garcia et al., 2012). The assay yields a concentrated violet solution that is constant at room temperature without the direct light exposure. In this study, radical scavenging activity of KH was determined using Multiskan GO microplate spectrophotometer at 517 nm against DPPH radical as described by Beretta et al. (2005). Following preparation of DPPH solution through dissolving 2 mg of DPPH in 100 mL of ethanol, 1 mL of ethanolic KH solution was mixed to 2 mL of DPPH solution (Figure 2). The reaction mixture was shaken vigorously using hands for yielding the good mixture and later stored without exposure to light for 30 minutes at a room temperature. Subsequently, the absorbance of the mixtures was recorded. The scavenging activity of KH via DPPH radical was quantified based on the equation given below:

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{(Abs control-Abs sample)}}{\text{Abs control}} \times 100 \quad (1)$$

where Abs control denotes the absorbance of DPPH radical and ethanol, and Abs sample indicates the absorbance of DPPH radical and honey or ascorbic acid. The analysis was conducted three times.

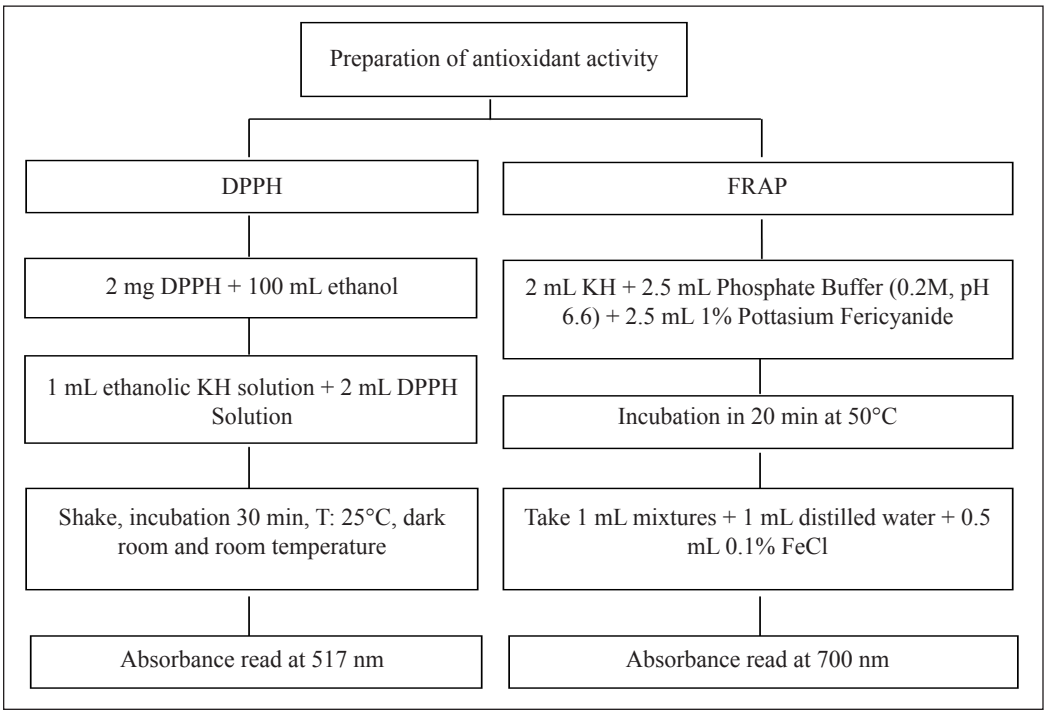

Figure 2. Flowchart representing steps in DPPH and FRAP analyses

Ferric Reducing Antioxidant Power (FRAP). FRAP is a parameter, which is used to measure the antioxidant or reductant activity in a sample. Total antioxidants were assessed by the reduction of Fe^{3+} to Fe^{2+} (Yen & Duh, 1993; Ahmad & Abdullah., 2013). In this study the FRAP analysis was conducted by combining a mixture of 0.5g KH and 100 mL distilled water with 2.5 mL of phosphate buffer (0.2M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (Figure 2). The mixtures were incubated for 20 min under 50°C. After incubation, a total of 2.5 mL of 10% trichloroacetic acid were combined to the mixture. The 1 mL mixture was added with 1 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance of the solution was quantified at 700 nm via a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510). The FRAP activity of KH was expressed in absorbance unit.

pH and Color Intensity

The color intensity that represents the darkness value of honey was determined by using the method described in a previous study (Beretta et al., 2005). A 50% (w/v) honey solution

was prepared with warm water at 45 to 50°C. The net absorbance was determined using the Multiskan GO microplate spectrophotometer at 450 nm. The pH was measured using a pH meter, model LAQUA twin pH (HORIVA, Japan).

Statistical Analysis

The significance between means were analyzed using one-way analysis of variance (ANOVA) as well as Tukey's multiple-comparisons analysis. The significant level was set at $P \leq 0.05$. The correlation between every measurement was examined via Pearson correlation coefficient.

RESULTS AND DISCUSSION

Total Phenolic Content and Total Flavonoid Content

Table 2 demonstrates the amount of TPC and TFC constituents in KH samples according to the standard curve of gallic acid and quercetin, correspondingly. The KH from the different geographical had different TPC and TFC values. The finding of this study is in agreement with a previous study that reported the significant differences between TPC and TFC of honey samples that were collected from Kedah and Johor (Ranneh et al., 2017). On that account, Silva et al. (2013) reported that flavonoids and polyphenols detected in honey resulted in superior antioxidant activity.

The findings indicated that the TPC varied significantly ($P \leq 0.05$) among the geographical locations in Malaysia. The TPC value was ranged from 3.045 to 9.370 mg GAE/100g FW. KH from south Malaysia demonstrated the highest TPC followed by the north, Sarawak, Sabah, east and central Malaysia. This significant differences could be due to the variations in the source of pollen surrounding the cultivated area. A research

Table 2
The phytochemical contents, antioxidant activities, color intensity and pH in KH from various regions in Malaysia

Regions	TPC (mg GAE/100g FW)	TFC (mg QUE/100 FW)	DPPH (%)	FRAP (abs)	Color Intensity (abs, mAU)	pH
South	$9.37^{a*} \pm 0.23$	$14.44^{a} \pm 0.18$	$2.77^{c} \pm 1.02$	$0.22^{a} \pm 0.002$	$0.30^{a} \pm 0.02$	$5.13^{ab} \pm 0.09$
North	$6.15^{b} \pm 0.5$	$9.45^{b} \pm 0.18$	$18.65^{b} \pm 2.10$	$0.22^{b} \pm 0.002$	$0.23^{b} \pm 0.01$	$5.20^{ab} \pm 0.16$
Sarawak	$5.49^{bc} \pm 0.50$	$8.72^{c} \pm 0.12$	$19.25^{b} \pm 7.11$	$0.22^{b} \pm 0.003$	$0.21^{c} \pm 0.01$	$4.25^{d} \pm 0.19$
Sabah	$5.17^{c} \pm 0.05$	$7.29^{d} \pm 0.14$	$1.98^{c} \pm 0.45$	$0.20^{c} \pm 0.02$	$0.21^{c} \pm 0.02$	$4.58^{dc} \pm 0.17$
East	$3.79^{d} \pm 0.50$	$5.24^{e} \pm 0.18$	$30.36^{b} \pm 2.28$	$0.19^{c} \pm 0.02$	$0.13^{d} \pm 0.003$	$5.50^{a} \pm 0.36$
Central	$3.04^{d} \pm 0.1$	$3.63^{f} \pm 0.25$	$44.05^{a} \pm 11.04$	$0.19^{c} \pm 0.002$	$0.09^{e} \pm 0.003$	$4.8^{bc} \pm 0.22$

*Means with the same letter are not significantly different at $P \leq 0.05$ by using Tukey test

study by Kroyer and Hegedus (2001) found that flavonoids were among the important forms of polyphenols, which existed in pollen composed by honeybees. Nijveldt et al. (2001) demonstrated that this category of bioactive compounds functioned as a superior antioxidant that facilitated scavenging activity of free radicals. Additionally, the authors concluded that flavonoids stabilized and neutralized reactive oxygen species to generate a reduced reactive radical.

The results also revealed that the TFC demonstrated a significant difference between different geographical locations with the values ranged from 3.63 to 14.44 mg QUE/100g FW. The highest TFC was exhibited by the south and the lowest was from the central. KH samples were belonging to dissimilar geographical and botanical origins, which could be the reason for the differences. These findings are in agreement with the past studies by Biluca et al. (2016), Silva et al. (2013), Da Silva et al. (2013), Silici et al. (2010), Lachman et al. (2010) and Al et al. (2009), which demonstrated that the values of TPC and TFC of honey were influenced by the geographical floral origins.

Antioxidant Activity

Table 2 demonstrates that there was a significant difference between DPPH and FRAP activities as affected by geographical locations. KH from central Malaysia possessed the highest ability to scavenge DPPH radicals with inhibition of DPPH by 44% ($P \leq 0.05$) followed by the east, Sarawak and north, whereas the KH originated from the south and Sabah demonstrated low in DPPH inhibition with values lower than 5%. The results obtained was contradicted with Chan et al., (2017) who found that KH originated from the east coast region had the highest scavenging activity against DPPH radicals with scavenging percentage of 44.12-79.99%. Similarly, Tuksitha et al. (2018) found that DPPH assay contents of the three different stingless bees species (*Geniotrigona thoracica*, *Heterotrigona itama* and *Heterotrigona erythrogastra*) were ranged from 17.0 ± 7.5 to 47.4 ± 3.2 (%). Meanwhile, Abu Bakar et al. (2017) revealed that *Heterotrigona itama* honey collected in Jasin, Melaka exhibited the highest levels of DPPH assay with the values of 97.30 ± 0.84%.

In the present study, FRAP activity for KH from the south of Malaysia demonstrated the highest antioxidant potential while KH from the central region of Malaysia obtained the lowest values ($P \leq 0.05$). The coincidence of KH from the south of Malaysia having the highest TPC, TFC, FRAP highlighted the contributions of these bioactive compounds in antioxidant activity. The roles of TPC and TFC in antioxidant activity of stingless bee honey have been studied extensively (Silva et al., 2013; Ibrahim et al., 2016: Kek et al., 2017). Therefore, evidence has confirmed that TPC and TFC play crucial role in the antioxidant activity of KH, with the effect of the geographical of the nectar source in determining the bioactive contents.

pH and Color

The differences with regard to pH and color among the honey varies according to the type of bee species, region, the season of collection and type of floral sources. The Malaysian Standard (2017) had established a standard pH range between 2.5 to 3.8 for stingless bee honey. Nonetheless, a review by Nordin et al. (2018) found the pH of stingless bee honey varied between 3.15 to 6.64. Furthermore, authors concluded the honey of *Melipona scutellaris* from Brazil had the lowest pH value of 3.15 (Marchini et al., 1998). Meanwhile, the honey of *Melipona quadrifasciata* from Brazil had the highest pH value (Carvalho et al., 2009).

Table 2 demonstrates that the KH from the north of Malaysia had the highest pH value in which the pH values of all the KH samples were ranged from 4.25 to 5.5. In a similar study, Syam et al. (2016) reported that pH of *Trigona* honey from Masamba, Indonesia was 3.34 while Chanchao (2009) revealed that pH honey of *Trigona Laviecep* from Thailand was 3.37. On the other hand, Boorn et al. (2010) found that pH of *Trigona carbonaria* honey from Queensland was 3.85. The aforementioned findings reveal that KH is acidic, which contributed to the sourness of KH. Thus, the antimicrobial capacity of KH may be derived from pH values.

Moniruzzaman et al. (2013) reported that color intensity of honey was a consistent parameter that specified the existence of pigments that had antioxidant activities including carotenoids and flavonoids. Solayman et al. (2016) stated that the color of honey could differ from straw yellow to nearly black as it was affected by the mineral, pollen and phenolic contents of the honey. This study has discovered that color intensity values of the honey from the south of Malaysia had the highest values followed by the north, Sarawak, Sabah, east and central of Malaysia (Table 2). The color intensity of each KH samples varied between 0.0925 mAU to 0.30125 mAU. Recently, Nordin et al. (2018) indicated that color varieties of stingless bee honey across the different countries were 26 Pfund to 150 Pfund, which were detected via a photometer. Furthermore, the authors reported the highest intensity of color, which was detected in *Tetragonisca angustula* honey from Brazil and the lowest intensity of color, which was detected in *Melipona ilota* from Peru.

A study by Bertoncelj et al. (2007) reported that color of honey was significantly affected by its geographical starting point. Kek, et al. (2014) demonstrated that KH had the highest color intensity compared to Tualang, Gelam, Pineapple, Borneo and commercial honey in Malaysia. Apart from geographical and species, the variations in the color intensity of the honey might be influenced by particular polluting pigments originating from handling, processing, and storage processes, or from biochemical reactions throughout honey maturation (Beretta et al., 2005). Nevertheless, there is a lack of standard available for honey with regard to pH and color.

Correlation Between Phytochemical Properties, Antioxidant Activity and Color

The correlation analysis revealed that there was a strong, negative and significant correlation between TPC and TFC with DPPH activity (Table 3). As such, this implies that the TPC and TFC increased inversely with DPPH activity. Furthermore, this indicates that the increase in TPC and TFC of KH resulted in the decrease in DPPH activity. The decrease in DPPH activity is correlated with the ability of the KH to neutralize the free radical of DPPH from being oxidized by reactive oxygen species (Nurdianah et al., 2016). The high content of TPC and TFC might not contribute to the strong antioxidant activity. These results are consistent with Chan et al. (2017), which demonstrated that KH had high TPC and TFC but low DPPH activity. Hence, this indicates that higher TPC and TFC may not lead to high DPPH activities.

Table 3
Correlation between phytochemical contents (TPC and TFC), antioxidant activities (DPPH and FRAP) and color intensity in KH collected from various regions in Malaysia

	TFC	TPC	DPPH	FRAP	Color Intensity
TFC	1				
TPC	0.97***	1			
DPPH	-0.80***	-0.73***	1		
FRAP	0.81***	0.81***	-0.79***	1	
Color Intensity	0.96***	0.94***	-0.83***	0.82***	1

*** Correlations are significant at $P \leq 0.001$

The findings also indicate that TPC and TFC correlated positively with FRAP activity. This implies that the TPC and TFC increased proportionally with the FRAP activity. These findings are consistent with Chan et al. (2017), which demonstrated that stingless bee honey had higher TPC and TFC contents, thus leading to high in FRAP activity. The strong FRAP activity possessed by KH is related to the potential of the KH in reducing Fe^{3+} to Fe^{2+}. A correlation between TPC and antioxidant activity in the KH was reported by other studies (Duarte et al., 2012; Sousa et al., 2016; Ranneh et al., 2017). Hence, polyphenols compounds could result in a greater antioxidant potential in KH. In addition, the correlation between KH and antioxidant was reported to be high due to the high content of ascorbic acid (Guerrini et al., 2009; Kek et al., 2014).

The color of KH was found to be positively correlated and significantly associated with TFC, TPC, and FRAP activities (Table 3). TPC and FRAP activity increased proportionately with the increase in the intensity of the darker colour of KH. The finding is in accordance to Saxena et al. (2010) who reported that color intensity has positive correlations (R values between 0.72 and 0.83) with antioxidant activities in honey samples. Higher absorbance value was associated with darker color in KH samples. The darker colour of KH could be

due to the higher content of TPC and TFC, which could increase the potential of KH as an antioxidant property. Nevertheless, the colour of KH demonstrated a negative correlation with DPPH activity.

The correlation between TPC and TFC with color intensity demonstrated that there were strong, positive and significant correlations with R>0.8. The results of this study are in agreement with previous findings by Moniruzzaman et al. (2013) who reported that color intensity of KH was a consistent parameter that could indicate the existence of pigments. On that account, this finding indicates that the antioxidant activities like carotenoids and flavonoids. Kek et al. (2014) also reported a similar result and concluded that the TPC of honey increased with color intensity.

CONCLUSION

The current study indicated that TPC, TFC, antioxidant activity, color intensity and pH of KH could be elucidated by the natural disparities of floral nectar sources and geographical regions. Honey from the Southern region of Malaysia presented a good level of phytochemical composition and antioxidant activity as compared to the north, Sabah, Sarawak, east and central of Malaysia. The strong and positive correlations were observed between TPC, TFC, antioxidant property and color intensity of KH samples. Thus, the aforementioned findings suggest that honey with darker color had greater TPC, TFC, and antioxidant property. The findings could provide some fundamental data related to KH that can be used as a reference for future research.

ACKNOWLEDGMENTS

The authors are thankful to Universiti Putra Malaysia for providing financial support under the Geran Putra research funding (GP-IPS/9633300).

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