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Malaysian stingless bee and Tualang honeys: A comparative characterization of total antioxidant capacity and phenolic profile using liquid chromatography-mass spectrometry

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ABSTRACT

This study aims to examine and compare the phenolic profile and antioxidant properties of two kinds of honey: Stingless bee honey (Kelulut honey) and Tualang honeys. Using liquid chromatography mass spectrometry, a total of eighteen phenolic acids and flavonoids have been identified in Kelulut honey samples. The phenolic content and total antioxidant capacity (three assays: DPPH, ABTS, and ORAC) for Kelulut and Tualang honeys were evaluated and statistically estimated. There was a significant ($p < 0.05$) phenolic content (228.09 ± 7.9–235.28 ± 0.6 mg gallic acid equivalent per kg) and flavonoid content (97.88 ± 10.1–101.5 ± 11.4 mg catechin equivalent per kg) in Kelulut honey samples. The Kelulut honey samples have demonstrated a significantly stronger antioxidant capacity than Tualang honey samples. The correlations between antioxidant results and polyphenols content were found to be statistically significant ($P < 0.05$, $P < 0.01$). This research is the first to report data on phenolic profile and total antioxidant capacity of Kelulut honey. Our data suggest that Kelulut honey has prominent medical properties and could be exploited as a natural nutraceutical to treat free radical associated diseases.

1. Introduction

It has been well established in the literature that Reactive Oxygen Species (ROS), due to cellular metabolic reactions, are naturally formed in living organisms. The high concentration of radical species, including superoxide radical ($O^{\cdot -2}$), hydrogen peroxide (H_2O_2), Nitric oxide radical (NO·), hydroxyl radical (·OH) and peroxyl radical (ROO·) has recently proved to induce antagonistic modifications in cell component (D'Autréaux & Toledano, 2007; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). The occurrence of any negative functional alterations and/or destructive actions against cell components, such as protein, lipid and nucleic acid could also lead to various pathological conditions, including cancer, arthritis, neurological disorders, atherosclerosis, diabetes and hypertension (Cominelli, 2004; Dröge, 2002; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). Similarly, the low levels of antioxidant and/or high levels of free radicals have been reported to result in chronic diseases and oxidative stress damage (Halliwell, 2011; Keen, 2001). Research has attempted various preventive and therapeutic approaches to manipulate these chronic diseases. There has been a particular interest in improving and implementing new natural agents that combat oxidative stress-related diseases (Nimse & Pal, 2015). For example, a considerable number of epidemiological studies has emphasized the importance of diet-rich in antioxidant that are thought to have a role in longevity and increased healthy features (Chang, Alasalvar, & Shahidi, 2016; Kamiloglu et al., 2016; Zhang & Tsao, 2016).

It is quite common that bee honey has been a preferable natural therapy for wound healing and intestine-related diseases since centuries (Bogdanov, 2014, pp. 1–24). The use of honey in treatments has recently been termed as an apitherapy (Ghosh & Playford, 2003). Several studies have indicated that the scavenging and redox properties of natural honey are due to its content of various bioactive compounds; namely, phenolic acids, flavonoids, vitamins, and enzymes (Erejuwa, Sulaiman, & Ab Wahab, 2012). However, the geographical and botanical areas as well as the type of bees play a vital role in determining the biological composition of honey as well as the total antioxidant

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Abbreviations

ROS	Reactive oxygen species
LC-MS	Liquid chromatography-mass spectrometry
KH	Kelulut honey
TH	Tualang honey
TPC	Total phenolic content
TFC	Total flavonoid content
UHPLC	Ultra-High Performance Liquid Chromatography
DPPH	1,1-diphenyl-2-picrylhydrazyl
ABTS	2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid)
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
ORAC	Oxygen radical absorbance capacity

capacity (TAC) (Erejuwa, Sulaiman, & Wahab, 2012). The variety of flower species and its abundance all year round in Malaysia is thought to be the main source beyond the availability of different kinds of bee honey. Tualang honey (TH) and Kelulut honey (KH) are believed to be the most famous.

The TH, a tropical multifloral rain forest honey, is produced mainly by *Apis dorsata* (the rock bee), whereas KH is produced by *Trigona,* a stingless bee (Boorn et al., 2010). In Malaysia, these two kinds of honey have become very famous for being taken for different traditional treatment purposes (Barakhbah, Anisah, & Agil, 2007). Being gathered from plants' nectar, modified and stored by different types of honey bees, as well as being rich in polyphenols, there is an increasing number of studies attempting to figure out the biological composition of these two types of honey today (Abd Jalil, Kasmuri, & Hadi, 2017; Barakhbah et al., 2007; Bogdanov, 2014, pp. 1–24; Boorn et al., 2010). More recently, there are research reports that have found various benefits for Tualang honey, for being particularly anti-inflammatory (Othman, 2012), anti-microbial (Alvarez-Suarez et al., 2010), anti-oxidant (Barakhbah et al., 2007), and anti-ageing (Al-Rahbi et al., 2014). These therapeutic activities have been strongly attributed to the phenolic acid and flavonoids content of Tualang honey. More importantly, the polyphenolic compound accompanying its biological activities makes it of a particular interest from the nutritional and pharmacological viewpoints (Ghosh & Playford, 2003). Nonetheless, there is still much lacking about the phenolic profile and antioxidant capacity of KH.

Stingless bee honey (KH) is thought to have a higher content of polyphenol than any other kind of honey (Biluca, Braghini, Gonzaga, Costa, & Fett, 2016; Kek, Chin, Yusof, Tan, & Chua, 2014). This is likely attributed to the small size of *Trigona*'s body that helps her stretch itself inside a bigger number of flowers, and hence diversifying the bioactive compounds collected and put in the make-up of honey. It is presumed that the periodical monitoring of Total Antioxidant Capacity (TAC) as well as the polyphenols profile of KH and TH are crucially useful for health care and product quality insurance. Although previous studies have used several methods to measure TAC, they did not have measurements for more many samples together, and hence they did not have one unified report of their results. The development of a more efficient method for measuring TAC and a validation using 96-well microplate makes this study particularly important (Kambayashi et al., 2009). This study aims to examine the identification and quantification of polyphenols profile in KH and TH samples and to chart the differences between the antioxidant properties for KH and TH with known synthetic antioxidants *in vitro*.

2. Materials and methods

2.1. Honey samples

Two samples from *Tualang* honey (TH) and *Kelulut* honey (KH) were used. The two types of honeys, which are respectively produced by the *Apps dorsata* bees and *Trigona* bees, were supplied by two different local bee honey collectors. The first batches of TH and KH were collected from the forest of Kedah state, whereas the second batches were collected from the forest of Johor Bahru state (Table 1). The samples were all stored in sterile air-tight-glass bottles at 15 °C to rule out any moisture absorption during the time between their collection and analysis.

2.2. Chemicals

All the solvents and reagents used in this research were from analytical grade as well as DPPH (1,1-diphenyl-2-picrylhydrazyl). The gallic acid and catechin were purchased from Sigma-Aldrich, Chemicals, Co., USA. The ABTS and Oxygen Radical Absorbance Capacity (ORAC) kits were purchased from Zen-Bio, Inc., USA.

2.3. Proximate analysis

To identify the content of ash, moisture, protein, fat and carbohydrate in honey samples, we used proximate analysis according to the official analysis methods from the Association of Official Analytical Chemists (AOAC, 2005). All tests were carried out in triplicate.

2.4. Determination of vitamin C

Vitamin C (i.e., Ascorbic Acid) in all honey samples is usually determined using one previous method that has become a standard baseline in some studies (Ciulu et al., 2011), though it sometimes included minor modifications. Accordingly, vitamin C was quantified using a reversed phase high-performance liquid chromatography (HPLC) (Agilent 1100, Palo Alto, USA), and was connected with UV detector. The stock solution was prepared by dissolving 2.5 mg vitamin C into 50 ml of phosphate buffer (1M, pH 5.5) and 4 ml of NaOH (3 mol/L) in a 100 mL in a volumetric flask. Following that, 2.5 g of honey sample was dissolved of HPLC-water. Before injection, the honey solution was filtered through filter paper Whatman #4 (0.45 μm). A reversed-phase C18 column (Alltech, Licosphere, United States) (250 mm × 4 mm, 5 μ m I.D) was used for the separation of mobile

Table 1
Investigated honey samples.

SI No	Sample Code	Traditional and Scientific name	Floral and Bee type	Local and scientific name of trees	Time of collection	Region
1	TH1	Tualang Honey (Jungle honey)	Multifloral (*Apps dorsata*)	Tualang tree (*Kompassia excelsa*)	September 2015	Kedah, North of Malaysia
2	KH1	Kelulut honey (Stingless bee honey)	Monofloral (*Trigona*)	Forest Mangrove (*Acacia Mangium*)	September 2015	Kedah, North Malaysia
3	TH2	Tualang Forest honey	Multifloral (*Apps dorsata*)	Tualang tree (*Kompassia excelsa*)	May 2016	Johor Bahru, South Malaysia
4	KH2	Kelulut (Stingless bee honey)	Multifloral (*Trigona*)	Forest Mangorve (*Acacia Mangium*) Rambutan (*Nephelium lappaceum*) Longan (*Dimocarpus longan*) Belimbing (*Averrhoa carambola*)	May 2016	Johor Bahru, South Malaysia

phases solution A (trifluoroaceticacid (0.025%v/v)) and solution B (acetonitrile(100%v/v)) at constant flow rate 0.5 ml/min. The sample injection volume was 15 μL.

2.5. Total phenolic content

The total phenolic content in honey samples was determined colorimetrically using Folin-Ciocalteu reagents with slight changes, as was used by Beretta, Granata, Ferrero, Orioli, and Facino (2005). Also, 2 g of honey was diluted with 20 ml of HPLC-grade water, and then 200 μL of honey solution was mixed with 3 mL of 10% diluted Folin-Ciocalteu reagent. After 90 min of keeping in dark at room temperature, the absorbance was read at 750 nm using a UV spectrophotometer (PG Instruments Ltd, Leicestershire, UK). Serial concentration of Gallic acid was used to draw the standard calibration curve (0.02, 0.04, 0.06, 0.08, 0.1, 0.12 mg/ml; $r^2 = 0.994$). The results were the mean value of triplicate date that given in mg of Gallic acid per 1 kg of honey.

2.6. Total flavonoid content

The total flavonoid content was determined using our previous method (Ali, Ranneh, Ismail, & Esa, 2015), but with the inclusion of a slight modification. This included 1 ml of honey sample (0.1–0.4 g/ml) that was added to solution (2%) of aluminum chloride. After incubating for 10 min at 25 °C, the abosrbance of the mixture was measured at 430 nm using a UV-vis spectrophotometer. The serial concentration of (+)- catechin was used to draw the standard calibration curve (0.02, 0.04, 0.06, 0.08, 0.1, 0.12 mg/ml; $r^2 = 0.994$). The results were the mean value of triplicate date that given in mg of (+)- catechin acid per 1 kg of honey.

2.7. LC-MS/MS analysis

Each peak of honey samples separated using LC-ESI-MS/MS was carried out using AB Sciex 5500Q Trap LC/MS-MS system consisting of degasser, binary pump, auto sampler and column heater. The column vent was coupled to an Agilent 1290 series UHPLC mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) equipped with an ESI ion source. Data acquisition and mass spectrometric evaluation were performed in the laboratory computer of our institute using Sciex Analyst version 1.5 software. For chromatographic separation, a reversed phase C18 column (Phenomenex Synergi Fusion) (100 mm × 2.1 mm, particle size 3 μm) was used with work temperature 35 °C (Waters, MA, USA). The injection volume was 10 μL. The mobile phase solvents contained solvent A, which consisted of water with 0.1% formic acid and 5 mM ammonium formate, and solvent B, which consisted of acetonitrile with 0.1% formic acid and 5 mM ammonium formate. The following gradient run program was applied: 5%–95% B: 0.01–10.0 min, holding for 2 min and back to 10% B in 0.1 min and re-equilibration for 3 min. The overall flow rate was 250 μl/min. The following operating conditions were used during all MS experiments: for (ESI) Turbo interface operating was in a negative mode that has been proven to be more selective and efficient in characterizing phenolic compounds (Ramirez, Zambrano, Sepúlveda, & Simirgiotis, 2014), the capillary voltage was set to 4.5 kV, the drying temperature to

Table 2
Proximate analysis, Total polyphenols and Total Flavonoid content of investigated honey samples.

Parameter	TH1	KH1	TH2	KH2
Energy (kcal/100 g)	316.66 ± 1.52^a	275.3 ± 2.08^b	312 ± 2^a	277.3 ± 2.1^b
Moisture (g/100 g)	24.8 ± 0.36^b	29.8 ± 0.9^a	22.96 ± 0.89^b	30.42 ± 0.68^a
Ash (g/100 g)	0.163 ± 0.052^c	0.386 ± 0.01^a	0.309 ± 0.01^b	0.293 ± 0.03^b
Carbohydrate (g/100 g)	77.66 ± 1.52^a	68.1 ± 0.7^b	76.31 ± 0.41^a	68.56 ± 0.76^b
Protein (g/100 g)	0.519 ± 0.03^b	0.79 ± 0.01^a	0.686 ± 0.04^b	0.75 ± 0.03 ^{a}
Fat (g/100 g)	0	0	0	0
Vitamin C (mg/100 g)	32.27 ± 1.7^c	79.5 ± 0.65^b	26.72 ± 1.24^d	87.19 ± 0.75^a
Total Phenolic content (mg GAE/kg)	139.42 ± 13.7^c	228.09 ± 7.9^a	183.93 ± 24.1^b	235.28 ± 0.6^a
Total Flavonoid content (mg CE/kg)	64.72 ± 11.4^b	97.88 ± 10.1^a	66.98 ± 7.32^b	101.5 ± 11.4^a

The data are expressed as mean ± S.D. of triplicate analyses. Means with different letters were significantly different at the level of $p < 0.05$. GAE, Gallic Acid Equivalent; CE, Catechine Equivalent.

Table 3
Phenolic compounds identified in honey samples by LC-MS/MS with ESI. NI; Not Identified, RT; Retention Time, [*M*-H]$^-$ Molecular mass of honey extract on the loss of one proton measured by SIM.

Peak No	RT (min)	[*M*-H]$^-$ (Frag. MS^2 *m/z*)	Molecular formula	Compounds	Samples
1	0.92	169 (125)	$C_7H_6O_5$	Gallic acid	All
2	1.3	179 (135)	$C_9H_8O_4$	Caffeic acid	All
3	1.74	283.8 (241, 221, 179)	$C_{17}H_{16}O_4$	Caffeic acid phenethyl ester	KH2
4	2.09	197 (182, 147)	$C_9H_{10}O_5$	Syringic acid	KH1, KH2, TH2
5	2.75	289 (244.9, 175, 147)	$C_{15}H_{14}O_6$	Catechine	KH2, TH2
6	2.97	775 (715)	NI	Unknown	KH2, TH1, TH2
7	3.87	269 (251, 225, 205)	$C_{15}H_{10}O_5$	Apigenin	KH1, KH2, TH2
8	4.2	253 (209, 193, 178)	$C_{15}H_{10}O_4$	Chrysin	All
9	4.77	221	NA	Unknown	KH2, TH2
10	5.3	147 (118, 129, 102)	$C_9H_8O_2$	Cinnamic acid	All
11	5.67	162	NI	Unknown	TH2, KH2
12	6.5	165 (147, 119)	$C_9H_8O_3$	2-Hydroxycinnamic acid	All
13	7.6	285 (175, 151, 133)	$C_{15}H_{10}O_6$	Kaempferol	KH1, KH2, TH1
14	8.5	163 (119)	$C_9H_8O_3$	*P* coumaric acid	KH1, KH2, TH1
15	8.9	959 (913)	NI	Unknown	KH1, KH2, TH1
16	9.6	609 (301)	$C_9H_8O_3$	quercetin-3-*O*-rutinosid	KH1, KH2, TH2
17	10.2	614 (478, 452, 342)	NI	Unknown	All
18	12.4	137 (93)	$C_7H_6O_3$	4-Hydroxybenzoic acid	KH2

The phenolic compounds above were identified in the honey samples using LC-MS. This was achieved by comparing the mass spectrometric data with LC-MS library and literature data. Negative ionizations were used to detect the MS and fragment ions. Data shown are from a single experiment and are representative of 3 experiments.

500 °C, the nebulizer pressure to 40 psi and the drying gas flow to 100/min. The single ion monitoring (SIM) modality was performed to quantify the molecular ions of phenolic compounds. The SIM analysis, in this experiment, was scanned in the range of 100–1000 m/z for full scan and 50–1000 m/z for MS/MS scan. The mass fragmentation was done based on ACD/Labs advanced chemometrics mass fragmentation

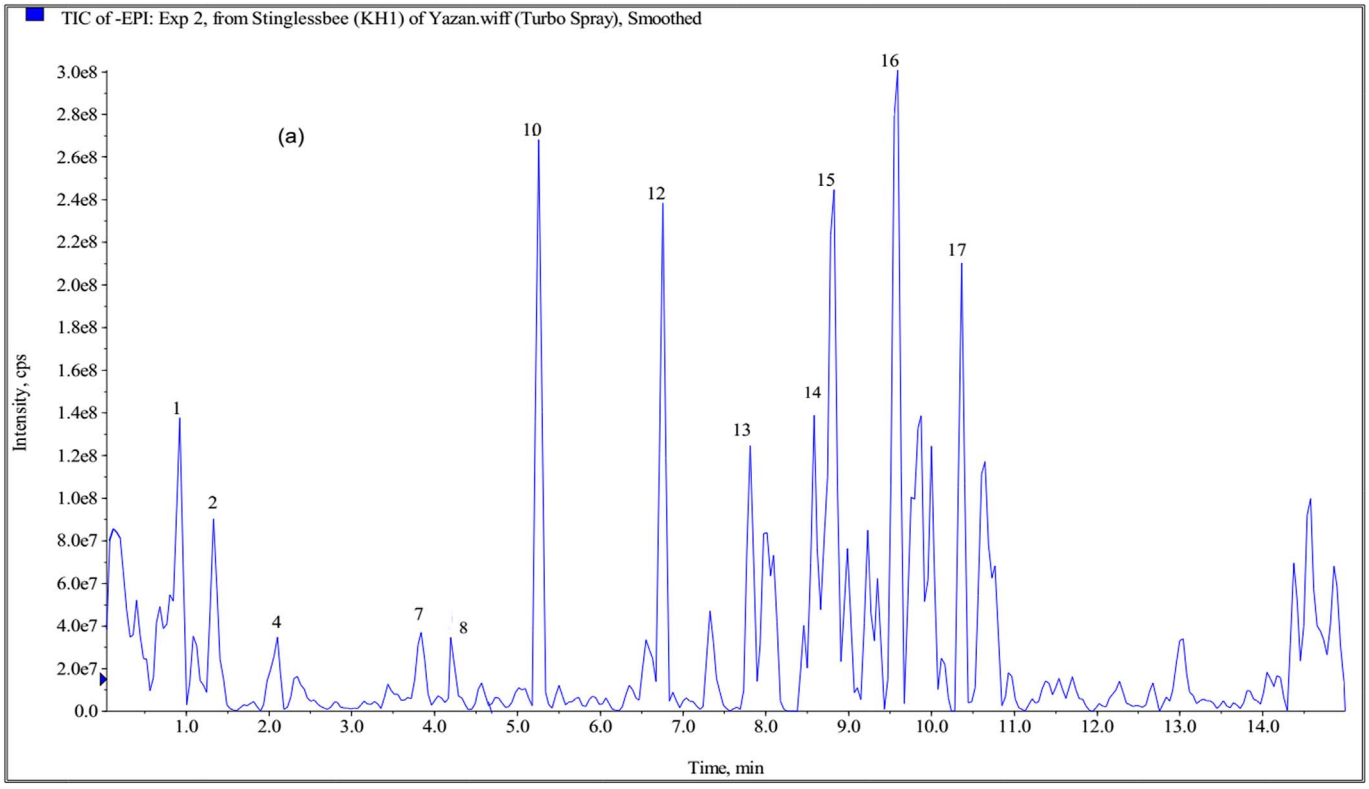


Fig. 1. LC-MS chromatogram at 280 nm of honey samples: (a) KH1; (b) KH2; (c) TH1; (d) TH2. The LC/MS-MS spectral data is listed in Table 3. The x-axis shows retention time in minutes, the y-axis shows the intensity.

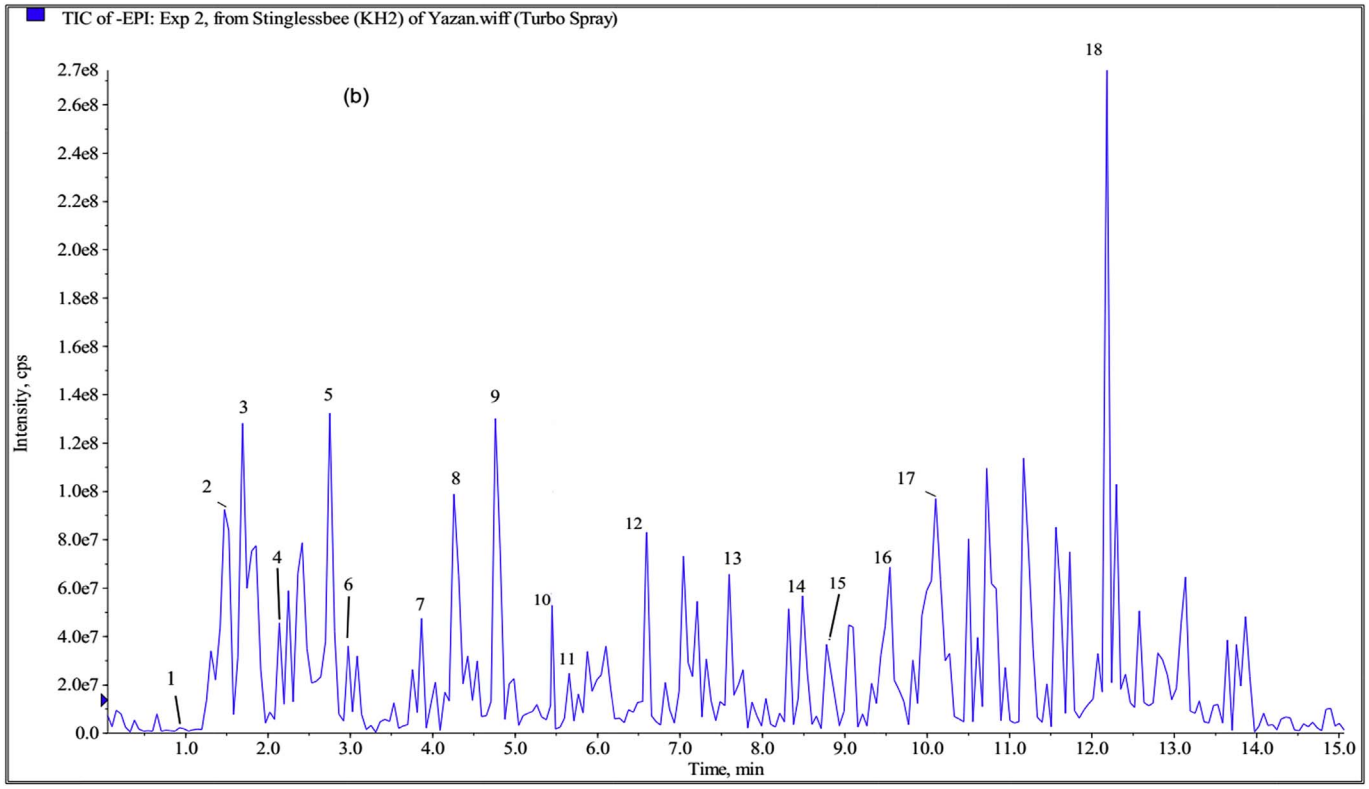


Fig. 1. (*continued*)

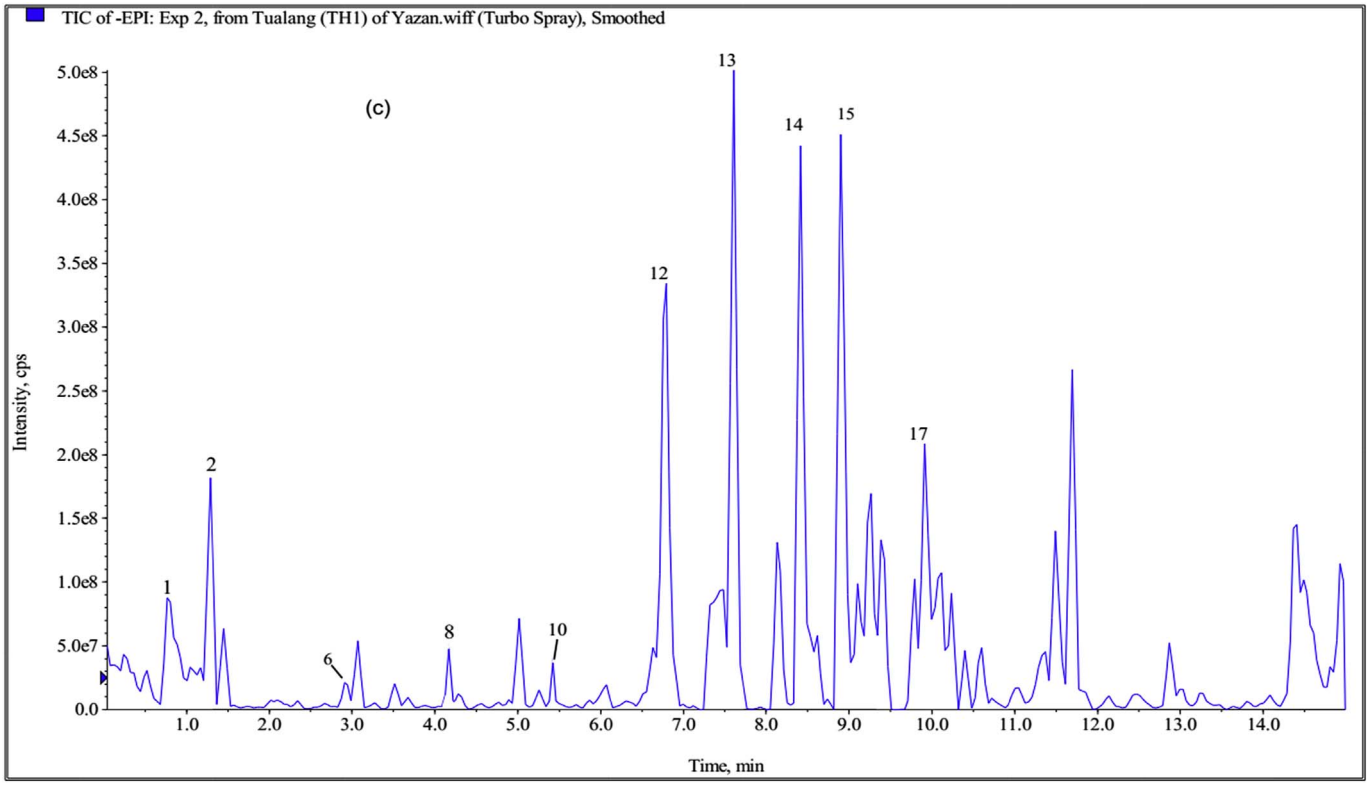


Fig. 1. (*continued*)

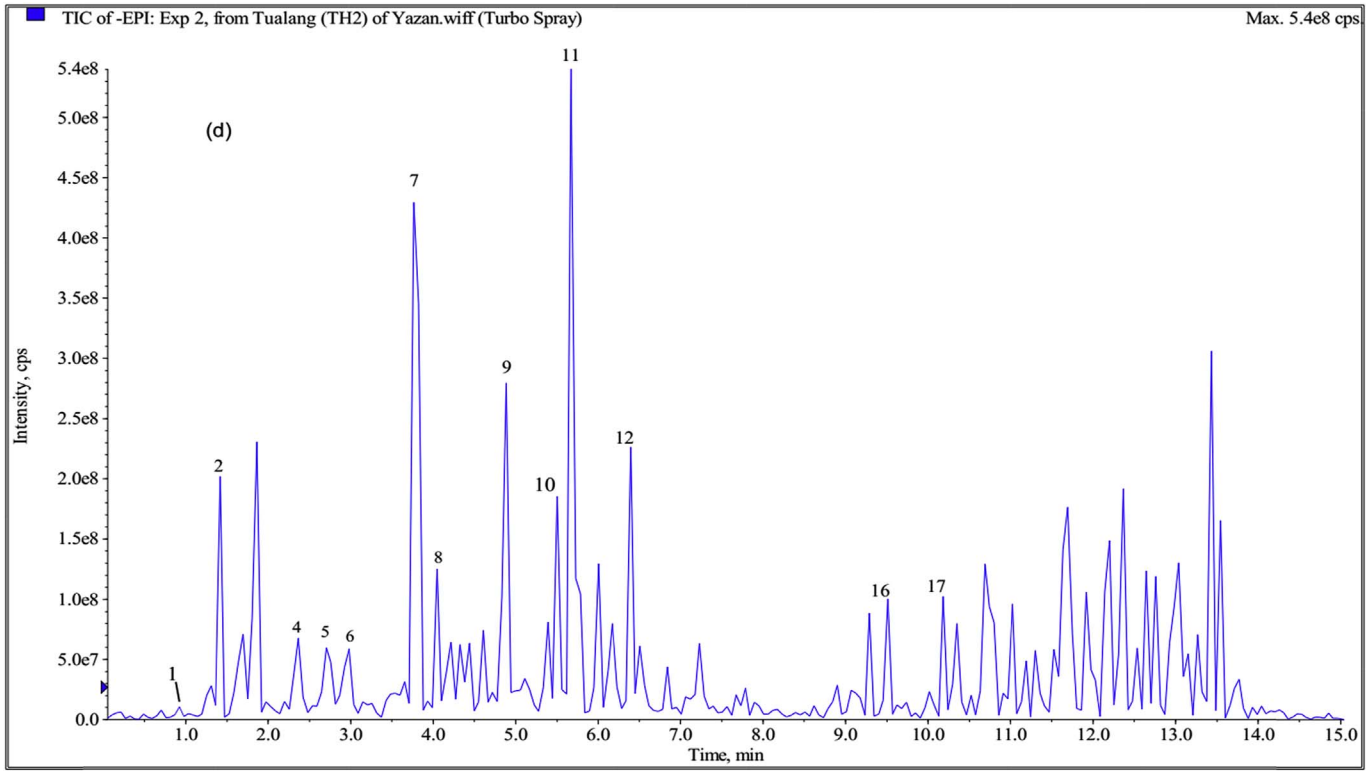


Fig. 1. (*continued*)

predictive software.

The phenolic acids and flavonoids were identified using a combination of liquid chromatography with mass spectrometry (ESI-LC-MS/MS) on the basis of their ultraviolet (UV) spectra, retention time, mass spectra and by comparing with our standard library information that includes 500 established compounds.

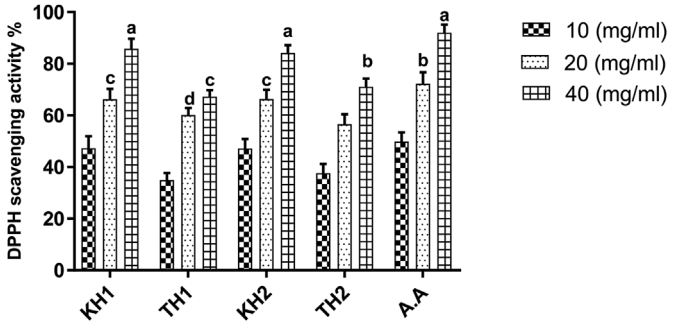


Fig. 2. Free radical scavenging activity of all honey samples compared with Ascorbic Acid (A.A) at 10, 20, 40 mg/ml using DPPH. Results are expressed as mean ± SD (n = 3). Bars having different letters are significantly different ($P < 0.05$).

2.8. Free radical scavenging activity

The determination of scavenging activity of honey samples against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was carried out using a different concentration (Beretta et al., 2005), with minor modifications. After preparing DPPH solution by dissolving 2 mg of DPPH in 100 mL ethanol, 1 mL of ethanolic honey solution and Ascorbic acid of a different concentration (10, 20, 40 mg/mL) were added to 2 mL of DPPH solution. The reaction mixture was hand-shaken for the purpose of a better mix and then kept for 30 min in a room temperature in the dark before measuring the absorbance of the mixtures at 517 nm with the use of a spectrophotometer. The scavenging ability of honey against DPPH radical was calculated by the following equation:

DPPH radical scavenging activity % = (Abs control – Abs sample) / Abs control X 100

where: Abs control is the absorbance of DPPH radical and ethanol, and Abs sample is the absorbance of DPPH radical and honey or Ascorbic acid. The experiment was performed in triplicate.

2.9. Total antioxidant capacity

2.9.1. ABTS assay

The total antioxidant capacity was obtained using ABTS assay, using the manufacturer's instructions (Zen-Bio, Inc., USA). After deluting the Myoglobin working solution with a dilution buffer, we added 10 μL of honey samples, Trolox standard and assay buffer to 10 wells of 96-well microplate. Then, we added 20 μL of myoglobin working solution and 100 μL of ABTS solution to start the reaction. After keeping the plate for 5 min at 25 °C. We stopped the reaction by appending 50 μL of stop solution in order to read the absorbance at 405 nm. The results were the mean value of triplicate date that expressed as micromoles Trolox equivalent per gram sample (μmolesTE/g).

2.9.2. ORAC assay

The ORAC assay was performed according to ZenBio instructions (Zen-Bio, Inc., USA). So, 150 μL of working fluorescein solution was injected to 60 wells of 96-well microplate. Then, 25 μL of Trolox (as a positive control), honey samples, or assay buffer (as a negative control) were added to individual wells of assay plate to be incubated for 10 min at 37 °C. The reaction was initiated by injecting 25 μL of AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride) working solution to each well. AAPH oxidized the fluorescein causing a decrease in fluorescence emission which was measured kinetically in a microplate reader (FLUOstar Omega) at excitation 485 nm and emission 528 nm. The data was taken for 30 min with one-minute intervals. The antioxidant capacity of honey samples was calculated by comparing the area under fluorescence curve (AUC) for each sample to the standard Trolox and were expressed as micromoles Trolox equivalent per gram sample (μmolesTE/g). The assays were performed in triplicate for each honey sample.

Table 4
Total antioxidant activity of Honey samples compared with Trolox at 25, 50, 100 μM using ABTS and ORAC assays.

Honey sample	Honey concentration (μM)	Total Antioxidant Capacity (μmoles TE/g) [a]	
		ABTS	ORAC
TH1	25	45.89 ± 4.37^{f}	22.28 ± 0.4^{h}
	50	120.25 ± 9.2^{e}	40.23 ± 2.18^{f}
	100	196.15 ± 7.41$^{b, c}$	75.47 ± 2.5^{c}
KH1	25	176.66 ± 17.6$^{c, d}$	29.56 ± 1.06^{g}
	50	201.8 ± 5$^{a, b}$	50.37 ± 1.9^{d}
	100	231.5 ± 6.1^{a}	89.44 ± 2.1^{a}
KH2	25	169.49 ± 18.7^{d}	30.62 ± 2.09^{g}
	50	208.64 ± 5.5$^{a, b, c}$	49.9 ± 1.8$^{d, e}$
	100	234.87 ± 2.9^{a}	83.72 ± 2.8^{b}
TH2	25	53.73 ± 4.3^{f}	26.97 ± 0.45^{g}
	50	129.35 ± 8.6^{e}	44.79 ± 1.3^{e}
	100	219.36 ± 24.3$^{a, b}$	48.69 ± 1.02$^{d, e}$

[a] Expressed as micromoles Trolox equivalent per gram of honey sample; values are mean ± S.D. of triplicate analyses. Means with different letters were significantly different at the level of $p < 0.05$.

2.10. Data analysis

The data was first subjected to Normality Test before analysis. The results showed that the data distribution was normal. We then calculated data for their mean value and standard deviation (SD). One-way analysis for variance (ANOVA) or Two-way ANOVA followed by Tukey's honestly significant difference (HSD) post hoc test was performed. The differences were considered significant at ($P < 0.05$) using SPSS for Windows. Version 18.0. Pearson correlation coefficient was used to determine the correlation between parameters studied in honey samples.

3. Results

3.1. Proximate analysis, total phenolic and total flavonoid content

Table 2 presents the proximal content of the stingless bee and Tualang honeys from north and south Malaysia (moisture, carbohydrate, protein, fat, ash and energy). The moisture, ash and protein content of KH1 and KH2 were significantly higher ($P < 0.05$) than TH1 and TH2. However, the carbohydrate content and Energy values of TH1 and TH2 were notably similar, and were the highest with values from 77 to 76 g/100 g and 316 to 312 kcal/100 g. Significantly, the content of vitamin C was in the wide range between 20 and 90 mg/100 g among all the studied honey samples. The highest amount was found in KH2 with a value of 87.19 ± 0.75 mg/100 g while the lowest was found in TH2 with a value of 26.72 ± 1.24 mg/100 g.

Table 2 also presents the level of phenolic and flavonoid content in honey samples that used the standard curve of gallic acid and catechin (R^2 = 0.999). The stingless bee honey samples KH1 and KH2 showed significant phenolic content with values of 228.09 ± 7.9 and 235.28 mg GAE/kg, respectively. The mean phenolic content of TH1 and TH2 ranged from 130 to 180 mg GAE/kg. The flavonoid content of all the samples ranged approximately between 60 and 100 mg CE/kg, but the highest percentage was in KH1 and KH2 followed by TH2 and TH1.

3.2. Identification of polyphenols in honey samples by LC- ESI-MS/MS

Based on the optimization conditions of LC-ESI-MS/MS, all honey samples were subjected to identify the polyphenols compounds. By searching in our standard library information (e.g. Peak retention

Table 5
Correlation matrix (Pearson correlation coefficients) between antioxidant activity, phenolic and flavonoid content.

	TFC (TH)	TFC (KH)	TPC (TH)	TPC (KH)	DPPH (TH)	DPPH (KH)	ABTS (TH)	ABTS (KH)	ORAC (TH)	ORAC (KH)
TFC (TH)	1									
TFC (KH)		1								
TPC (TH)	0.623		1							
TPC (KH)		0.686		1						
DPPH (TH)	0.922[a]		0.584		1					
DPPH(KH)		0.936[a]		0.607		1				
ABTS (TH)	0.482		0.76		0.623		1			
ABTS (KH)		0.711		0.422		0.82[b]		1		
ORAC (TH)	0.926[a]		0.287		0.824[b]		0.594		1	
ORAC(KH)		0.945[a]		0.584		0.99[a]		0.817[b]		1

TH = Tualang Honey, KH= Kelulut Honey.
TPC = Total Phenolic content.
TFC = Total Flavonoid content.
[a] Correlation is significant at the 0.01 level (2-tailed).
[b] Correlation is significant at the 0.05 level (2-tailed).

times, UV spectrum, [M-H] (m^2) and ESI-MS/MS data), a total of eighteen phenolic compounds, consisting of around eight phenolic acid compounds and five flavonoids, were investigated with range of 0.8–12.4 retention times (RT) in total ion chromatogram (See Table 3, Fig. 1(a) and (b), 1(c), and 1(d)). Peaks 1, 2, 3, 4, 10, 12, 14, and 18 referred to phenolic acid class while peaks 5, 7, 8, 13, and 16 belong to flavonoid class. The other five unknown compounds were not identified under the same conditions because they required other tools to identify them. There were significant differences found in the number of identified compounds of honey samples. As shown in Fig. 1 and Table 3, the gallic acid, caffeic acid, chrysin and 2-hydroxycinnamic acid were identified in all the honey samples, whereas the Caffeic acid chrysin and 4-hydroxybenzoic acid were only identified in sample KH2. Five compounds which are caffeic acid, Caffeic acid phenethyl ester, Cinnamic acid, 2-Hydroxycinnamic acid, and *P* coumaric acid are classified as hydroxycinnamic acid. It was clear that the identified compounds in sample TH1 was importantly lower than those detected in TH2, KH1 and KH2 (See Fig. 1).

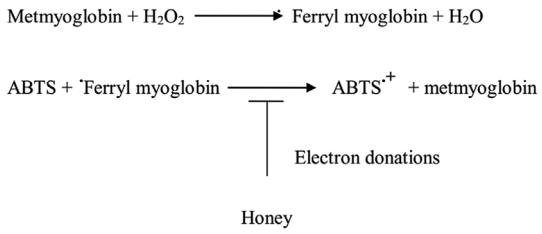
3.3. Free radical scavenging activity

The percentage inhibitions shown in Fig. 2 represent the free radical scavenging activity results for honey samples. There was a general decline in the percentages of DPPH radical between honey samples. The KH1 and KH2 samples (stingless bee honey) have exhibited the highest scavenging activity with a value of 90% and at concentration of 40 mg/ml, whereas the TH1 and TH2 samples have showed a lower inhibition of 70% and a similar concentration. Overall, these results demonstrated that the increase of scavenging activity of honey samples remains consistent with the increase of concentrations.

3.4. Total antioxidant capacity

3.4.1. ABTS

The oxidation of ABTS by ferryl myoglobin radical with H_2O_2 proceeded linearly as a function of time. ABTS activity was quantified by the decolourization of the $ABTS^{\cdot +}$, through measuring the reduction of the radical cation, as the percentage inhibition of absorbance is at 405 nm. The honey samples have shown a different rate of inhibitory effect against ABTS oxidation compared with the effect of Trolox. The highest TAC in honey samples has been noticed in KH2 sample ($P < 0.05$), followed by KH1, TH1 and TH2 as shown in Table 4. The equations below exhibit the proposed mechanism of actions of honey as a potent inhibitor towards ABTS free radicals in the reaction of the assay.



3.4.2. ORAC

The different concentrations of all honey samples (25, 50, 100 μM) were compared to the standard curve of Trolox for their ability to protect fluorescein from oxidation by ROS generated by AAPH. As demonstrated in Table 4, the antioxidant activity assessed by ORAC assay ranged between 20 and 85 μmol TE/g, with KH1 showing the highest antioxidant capacity (89 μmol TE/g) at concentration 100 μM, and with a significant difference ($P < 0.05$) compared to the honey samples TH1 and TH2. Finally, KH1 > KH2 > TH1 > TH2 represents the ranking of honey samples from the highest to the lowest antioxidant capacity.

4. Discussion

This study aimed to investigate two types of Malaysian honey; namely, Tualang and Kelulut honey. Our findings have indicated that there are significant differences between TH and KH, particularly in the proximate analysis, vitamin C content, total phenolic and flavonoid content, total number of phenolic compounds detected, and the total antioxidant capacity.

Proximate analysis is essential to determine the nutrient content which is important for health promotion and metabolic energy. As shown in Table 2, the differences in energy in honey samples were attributed to carbohydrate content, where the TH samples had the highest value. This value was consistent with similar results from previous studies (Erejuwa et al., 2012). The KH samples also showed a higher amount of protein and ash compared to TH samples. That was thought to be attributed to the floral type visited by the stingless bee (*Trigona*). Our findings were also compliant with previous proximal studies of stingless bee honey (B. A. Souza, Heard, Marchini, Díaz, & Tricio, 2006a, pp. 867–875). For example, the vitamin C value for TH was somehow similar to (Kishore, Halim, Syazana, & Sirajudeen, 2011), who compared TH samples with gelam, pineapple and Indian forest honey. However, the KH samples have exhibited a higher amount of vitamin C compared to other types of stingless bee honeys from

various countries (B. Souza et al., 2006b).

Since the bioactive actions of honey have been attributed to its phenolic and flavonoid content in previous studies, a high concentration of phenolic substances was recorded in our samples (Table 2). The total phenolic and flavonoid content of KH samples were significantly higher than those in the two reported studies on Malaysian honey samples (Aljadi & Kamaruddin, 2004), as well as more than those of Gelam and coconut honeys (Kishore et al., 2011). Additionally, there is evidence in the literature that the increased rate of colour intensity in *Trigona* stingless bee honey is usually associated with a high amount of polyphenols (Kek et al., 2014). However, this contrasts with similar evidence that reports that strawberry tree honey has the highest phenolic content that may reach to 789 mg GAE/kg (Beretta et al., 2005). A direct comparison between the findings of previous studies and the current results tends to be less logical, because there are methodological differences as well as material-related ones across each other.

Our results also showed, after inspecting the profiles of polyphenols in our honey samples, that using LC-MS/MS data analysis which was done under the conditions described above can distinguish each type of polyphenols based on the molecular weight (Table 3). The majority of identified polyphenols are classified under phenolic acid that explains the high phenolic content, while some of the flavonoid compounds are bounded to sugar moieties, such as (quercetin-3-*O*-rutinosid), that make them more soluble in water. It is more surprising that this is the first time in which Kelulut honey (stingless bee honey) has been investigated with LC-MS/MS analysis, where a total of 13 compounds were identified (See Fig. 1(b)). These compounds are thought to be the main content that made stingless bee honey (KH) a good source of antioxidant.

This study also assessed and compared the antioxidant potency for Tualang and Kelulut honey with the synthetic antioxidants (Ascorbic Acid and Trolox (vitamin E)) *in vitro*. Interestingly, our findings indicated that honey has a significant antioxidant property after comparing the antioxidant efficacy in synthetic antioxidants. In particular, the Kelulut honey (KH), as an antioxidant agent, was significantly better than Tualang honey (TH).

The DPPH assay offered a cost-effective and rapid assay in evaluating the radical scavenging activity of honey. DPPH result is usually expressed as an inhibition percentage, which means that Kelulut honey samples (KH1 and KH2) showed a significant free radical scavenging compared with Tualang honey samples. In a similar line of study, the Ecuadorian stingless bee honey showed a similar antioxidant potential (Guerrini et al., 2009). The correlation between the total phenolic and flavonoid content in our honey samples and DPPH was significantly positive (See Table 4).

In this study, we used two arrays of analytical methods to benchmark the antioxidant potency of stingless bee honey samples (KH) and Tualang honey (TH). ABTS assay measured radical scavenging activity by electron donation. When it was combined with ORAC assay that measures hydrogen atom transfer, a comprehensive analysis of total antioxidant capacity (TAC) was provided. A 96-well plate was used in the two assays (ABTS and ORAC). Previous research suggested that ABTS assay using microplate would help in cutting the cost to 25%, and hence increasing the analyzed sample and improving the analytical performance compared with other used methods (Kampa et al., 2002).

The results also showed that the oxidation of ABTS has been perfectly inhibited in the stingless bee honey, especially KH2, when compared with Trolox (See Table 4). The usage of ABTS assay-microplate in evaluating TAC in our results was to Kambayashi et al. (2009) As for ORAC assay, the stingless bee honey samples (KH) have demonstrated a significant scavenging activity of peroxyl radicals that are considered common in human body. In this respect, ORAC measurements could be biologically relevant (See Table 4).

This study also found a significant correlation between the polyphenols content of honey samples and antioxidant parameters (ABTS and ORAC, see Table 5). Accordingly, the stingless bee honey (KH) has a stronger antioxidant capacity than Tualang honey (TH). It was also notable that the demonstration of more polyphenols compounds in stingless bee sample (KH2) has contributed to a high antioxidant potential. However, the relationship between honey and antioxidant reported in previous studies is thought to be attributed to the high content of Ascorbic Acid (Guerrini et al., 2009; Kek et al., 2014).

Overall, the four honey samples were demonstrated middle differences in the results, however the exception was occurred in the KH2 sample which is produced by Malaysian stingless bee (*Trigona*) and comes from beekeepers in south of Malaysia. Therefore, it is a possible marketed product that could be used as a beneficial source of healthy compounds in food industry to enhance the satisfactory quality characteristics of food formulations throughout improving sensory aspects, inhibiting browning reaction, and supporting probiotic bacteria (Yousuf & Srivastava, 2017). Nevertheless, identifying the biochemical components in Malaysian stingless bee honey to enhance their commercial value as alternative food additives in poor communities and increase stingless bee honey production is recommended.

5. Conclusion

This study that we report here is the first, to our knowledge, that investigates the phenolic profile and antioxidant properties of Malaysian stingless bee honey (Kelulut honey). In screening Kelulut honey using LC-MS/MS, eighteen phenolic compounds were detected. Our results demonstrated that Kelulut honey has a high antioxidant capacity against a variety of reactive oxygen species when compared with Tualang honey. Furthermore, we noticed that there are several strong, positive correlations between the phenolic content and antioxidant test values in Kelulut honey. The results given in LC-MS data and *in vitro* antioxidant for Malaysian stingless bee honey make us reflect on the ethnomedical usages as well as the therapeutic values that could be useful in promoting better nutrition and preventing oxidative stress-related diseases. A further investigation of this natural product is still required, particularly the bioactive components that could be of an attractive source of nutraceuticals ingredients.

Authors' contribution

Yazan Ranneh has designed research, performed experiments, interpreted data and wrote the manuscript. Maryam Zarei has edited the manuscript and interpreted data. Abdah Md AKim supervised the work and helped in editing the manuscript. Hasiah Abd. Hamid and Huzwah Khazaai supervised the project and designed research. All authors read and approved the final manuscript.

Conflict of interest

The authors have no competing interests to disclose.

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