Trop J Nat Prod Res, September 2021; 5(9):1597-1605 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Phytochemical Investigation, Comparison and Characterization Study of Malaysian Stingless Bee Honey versus Jordanian Honey by LC-MS/MS

Nesrin Seder[1], Walid A. Rayyan[2]*, Wael A. Dayyih[3], Mohammad A. Al-Natour[4], Abu Bakar Mohd Hilmi[1]*

[1]*School of Biomedical Sciences, Faculty of Health Sciences, Universiti Sultan Zainal Abidin, 21300 Kuala Nerus, Terengganu, Malaysia*
[2]*Department of Medical Laboratory Analysis, Faculty of Science, Al-Balqa Applied University, Al-salt, Jordan*
[3]*College of Pharmacy and Pharmaceutical Sciences/ Mutah University; Alkarak – Jordan*
[4]*Pharmaceutical Medicinal Chemistry and Pharmacogonosy Department, Faculty of Pharmacy and Medical Sciences, University of Petra, Amman, Jordan*

ARTICLE INFO

Article history:
Received 10 May 2021
Revised 15 August 2021
Accepted 18 September 2021
Published online 02 October 2021

ABSTRACT

Phytochemical studies have revealed more than 200 distinct polyphenolic compounds present in honey alone. Stingless bee honey (*Trigona* honey) is naturally found in tropical and subtropical regions. This study aims to interrogate the physical parameters, polyphenolic content, and antioxidant characteristics of Malaysian *Trigona* honey in comparison with Apis honey (*Centaurea hyalolepis*) and Citrus honey from Jordan. The three honey types were subjected to phytochemical and chromatographic analysis to explore the differences in honey composition related to bees type and geographical location. The total phenolic content ranged between 288.09 and 663.19 mg GAE/kg of honey in the three honey types. *Trigona* honey had the highest phenolic content (663.19 mgGAE/kg) followed by *Centaurea hyalolepis* honey (471.87 mgGAE/kg), both of which were higher than Citrus honey (288.09 mgGAE/kg). *Trigona* honey showed an IC_{50} of 61.042 ± 0.45 mg/mL, whereas, *Centaurea hyalolepis* honey and Citrus honey had IC_{50} of 120.29 ± 1.64 mg/mL and 129.51 ± 4.3 mg/mL, respectively. Statistical analysis has revealed a significant negative correlation between the IC_{50} value for the three honey samples and the concentration of polyphenols ($p \leq 0.001$). Chromatographic analysis using LC-MS/MS showed a 28 and 42-fold difference in the polyphenolic content in *Trigona* over *C. hyalolepis* and Citrus honey, respectively. In conclusion, the diversity in the polyphenols contents and the high amounts of phytochemical compounds found in *Trigona* honey confers the antioxidant activity and there is no unique compound responsible for such activity over *C. hyalolepis* and Citrus honey.

***Keywords*:** *Trigona* honey, Phytochemical, LC-MS/MS, Polyphenols, Jordan.

Introduction

Honey is produced naturally by Apis sp. and Meliponini sp. bee worldwide.[1] Nutritional scientists consider honey as a constituent of the human diet as well as a remedy for several medical issues.[2,3] The nutritional and therapeutic values of honey are proportionally dependent on concentrations of carbohydrates, amino acids, and polyphenols present in honey.[4] Phytochemical studies have revealed more than 200 distinct polyphenolic compounds in honey including phenolic acids, flavonoids, flavonols, catechins, and cinnamic acid derivatives that possess biological and clinical importance.[1,5] The biological activity is dependent on the botanical ingredients in honey and the darker the honey, the more potent it is.[6] The broad spectrum of antimicrobial potential along with the presence of different therapeutic ingredients makes it useful in the treatment of several disorders such as in the treatment of gastrointestinal tract, neurological, ophthalmological, and fertility disorders.[7-11]

*Corresponding author. E mail: mhilmiab@unisza.edu.my; walid.aburayyan@uop.edu.jo
Tel: +962790272152

Citation: Seder N, Rayyan WA, Dayyih WA, Al-Natour MA, Mohd Hilmi AB. Phytochemical Investigation, Comparison and Characterization Study of Malaysian Stingless Bee Honey versus Jordanian Honey by LC-MS/MS. Trop J Nat Prod Res. 2021; 5(9):1597-1605. doi.org/10.26538/tjnpr/v5i9.12

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Additionally, cardioprotective, antioxidant, antilipidemic, anti-inflammatory and anticancer properties were reported for honey.[12] In the ancient manuscripts, honey and natural products were used as a treatment for wound infections in Pharaonic civilization, Arabic countries, and India.[13] The usage of honey in pharmacological preparations as a backbone for the treatment of wound infections with drug-resistant strains has been reported.[14,15]

Honey is entirely the sole natural concentrated form of sugar made up of plants nectar after being processed by bees of the Apidae family.[6] Two types of bee's phylum are present: honey bees and stingless honey bees. There are many species of Honey bees belonging to an Apini tribe of the Apis genus, while there are only three genera of stingless bees that are members of the tribe of Meliponini: Melipona, Scaptotrigona, and Trigona.[16,17] Widely, each honey type has a unique colour, taste, consistency, acidity, biological and therapeutic characteristics according to the floral, geographic origin of the honey, the mode of collection, and storage conditions.[18,19]

Recently, more attention is paid towards natural antioxidant-rich agents such as honey to antagonize oxidative stress-related disorders.[20] The activity of honey is exerted through the phenolic compounds owing to their antioxidant activity as they can scavenge free radical species and prevent the damage of living cells and reduces the oxidative damage of reactive oxygen species on cells.[21] Additionally, factors such as hyperosmolarity, acidity, the ability to produce hydrogen peroxide enhance the therapeutic activity of honey.[22]

Trigona honey is multi-floral honey stored in a cluster of small resin domes in nests of stingless bees which are naturally found in the tropical and subtropical regions. Stingless bee honey possesses majorly lower levels of sugars, higher acidity and moisture content, as well as, higher levels of antioxidants and biological activities than

Apis mellifera honey.[23] Stingless bee honey possesses versatile characteristics such as higher liquefactive texture as well as the unique sour taste and aroma. From the medical point of view, *Trigona* honey has been allocated as a natural product with powerful antibacterial activity and is useful for therapeutic purposes.[3,24,25]
This study aims to interrogate the physical parameters, polyphenolic content, and the antioxidant characteristics of bee honey compared with stingless bee honey from two different geographical locations. Three honey types (*Trigona*, *Centaurea hyalolepis*, and Citrus honey) were subjected to analysis to study the variation in honey composition related to the difference of the Apidae bees and the geographical location using HPLC MS/MS and other chemical tests.

Materials and Methods

Chemicals
The solvents and reagents utilized in the current study were analytical grade. Folin Ciocalteu reagent (FCR), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), aluminum chloride ($AlCl_3$), Gallic acid, sodium carbonate (Na_2CO_3), Ascorbic acid, and Rutin were acquired from Sigma-Aldrich, Belgium.

Honey samples
Three honey samples of different types (*Trigona*, Citrus, and *Centaurea hyalolepis)* were selected for the current study. *Trigona* honey is stingless bee honey reared in a virgin rainforest in the Kelantan state on the East Coast of Peninsula, Malaysia. The honey sample was supplied by Bee Haven center, Kelantan. Citrus honey is a sting bee honey reared in citrus farms for citrus honey, whereas *C. hyalolepis* honey was reared at wild *Centaurea hyalolepis* farms. Citrus honey and *C. hyalolepis* honey were supplied by Alanabtawi farms in Jerash, Jordan. The honey collection was conducted from March to May, 2019. Honey samples were stored in well-closed glass bottles in a dark place at 20^{o}C for further investigations.

Physical analysis
Colour intensity
To determine the colour intensity for the honey samples, a dilution of 50% (w/v) was applied using distilled water. Samples were homogenized, then centrifuged for five minutes at 3200 rpm. Using a spectrophotometer (Eppendorf, USA), the absorbance of honey samples was determined at wavelength of 635 nm, and the colour intensity was expressed using the Pfund scale.[26,27]

pH
The pH was measured using a pH meter (Jumo, Germany). Honey samples were diluted to 10% (w/v) using double distilled water. The measurements were obtained in triplicates.
Moisture content
A refractometric technique was adopted for the determination of moisture content of the honey samples, whereas honey samples refractive indices were measured at room temperature using a Fuzhou Lindian portable refractometer (China, Fuzhou, Jiangxi). Wedmore's table was adopted to calculate the percentage of moisture content corresponding to the corrected refractive index.[28]

Determination of total phenolic content
Total phenolic content (TPC) in honey samples was determined colorimetrically using the Folin-Ciocalteu method described by Ali et al., (2015)[29] with some modifications. Gallic acid was used as a standard; this colorimetric assay is based on the principle of the capability of phenolic substances to reduce Folin-Ciocalteu reagent (FCR) in the presence of sodium carbonate (Na_2CO_3) causing a colour change.
Accordingly, for each honey sample, a stock solution was prepared at a concentration of 0.1 g/mL using distilled water. An aliquot of 0.2 mL of the honey stock solution was pipetted into a test tube and mixed with 2.5 mL of 10% diluted Folin- Ciocalteu phenol reagent, shaken gently then kept in the dark for 5 min at room temperature. Then, 2.5 mL of 7.5% anhydrous sodium carbonate (Na_2CO_3) was added. After stirring, the prepared mixtures were kept in the dark for 90 min at room temperature. A UV/VIS spectrophotometer (Eppendorf, USA) was used to measure the absorbance at 760 nm. The calibration curve was drawn by preparing the serial concentration of Gallic acid solution ranging from 0.0125 to 0.2 mg/mL for quantification. The results were expressed as mg of Gallic acid equivalent per kg of honey (mg GAE/kg). All of the spectrometric measurements were read in three replicates and the average value was used in the calculations of total phenolic content.

Determination of total flavonoids content
The colorimetric assay described by Liu et al., (2009)[30] was followed to estimate total flavonoids content (TFC) in honey samples using aluminum chloride ($AlCl_3$).
Each honey stock solution was prepared at a concentration of 0.1 g/mL. An aliquot of 1 mL of each stock solution was pipetted into a test tube and mixed with 1 mL of a 10% aluminum chloride solution. Following incubation for 30 min at room temperature, the absorbance of the reaction mixture was measured at 425 nm using a UV/VIS spectrophotometer (Eppendorf, USA). The calibration curve was generated using a Rutin solution as a standard with serial dilution from 0.005 to 0.1 mg/mL. The results were expressed in mg of Rutin equivalent per kg of honey. The results were the mean value of three replicates used in the calculations of total flavonoids content (TFC).[31]

Determination of free radical scavenging activity
The antioxidant activity of honey samples was determined using the DPPH method proposed by Isla *et al*., (2011)[26] with minor modifications. This method is based on the fact that the 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radicals are reduced by antioxidants.[26]
According to this method**,** DPPH solution was prepared by dissolving 6 mg of DPPH in 300 mL of methanol (0.025 mg/mL), then 2 mL of this solution was added to each test tube containing 1 mL of honey solution at different concentrations ranging from 5-80 mg/mL for each honey type, as well as Ascorbic acid which was used as a reference (positive control). The reaction mixtures were vigorously hand-shaken, then incubated at 25^{o}C for 30 min in a dark place, then the absorbance was measured at 517 nm using a UV/VIS spectrophotometer (Eppendorf, USA). The following equation was used to calculate the scavenging ability of honey to the DPPH radicals:

$$\% \text{ Radical scavenging activity (\%RSA)} = ((A_0-A_1)/A_0) *100$$

Where A_0: is the absorbance of the control (DPPH solution) at 30 min , and A_1: is the absorbance of the sample at 30 min.
The concentration of honey sample required to reduce 50% of DPPH radicals was expressed as IC_{50} (mg/mL) which was calculated by the interpolation from the graph of %RSA against sample concentration.[33] The test was performed in triplicate.

Phenolic compounds identified by LC-MS/MS
The analysis process for the peak spectrum of honey samples that were separated independently by LC-ESI-MS/MS was conducted using the AB Sciex 5500Q Trap LC/MS-MS system. The system consists of four major components; degasser, binary pump, autosampler, and column heater. An Agilent 1290 series UHPLC mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) was connected to a column vent and supplied with an ESI ion source. The evaluation of mass spectrometric and data acquisition processes were executed using Sciex Analyst version 1.5 software. For chromatographic separation, the operating conditions and machine specifications are summarized in Table 1.
All MS experiments were conducted using the following operating conditions: negative mode for (ESI) Turbo interface operating was settled, the capillary voltage was set to 4.5 kV, whereas, temperature was raised to 500°C for drying, the drying gas flow to 100/min and the nebulizer pressure to 40 psi.
To specify and quantify the molecular ions of phenolic compounds, a single ion monitoring (SIM) modality was performed. Through performing SIM analysis, a full scan in a range of 100–1000 m/z was

conducted followed by scanning a range of 50–1000 m/z for a specific MS/MS scan. To determine the mass fragmentation, the predictive software provided by ACD/Labs advanced chemometrics mass fragmentation was applied. The identification of phenolic acids and flavonoids was determined using a consolidation of ESI-LC-MS/MS based on their ultraviolet spectra and by comparing with library information containing more than 500 known compounds and literature.

Statistical analysis

The measurement of analytes was performed in triplicates then the results were expressed as the mean with standard deviations (SD). The one-way analysis of variance (ANOVA) was applied to calculate the significant differences between samples. To establish the correlations between the results, Pearson's correlation coefficient (r) was applied in bivariate linear correlations. Statistical analysis was performed using SPSS version 25 software.

Table1: Chromatographic conditions of the HPLC MS/MS.

HPLC Conditions	Pump Flow Rate	Auto-sampler Injection Volume	Auto-sampler Temperature	Column Oven
	250 µL/minute	10.00µL	10.0^{o}C	35.0^{o}C
Chromatography	Mobile Phase		A mixture of solvent A, consisting of 0.1% formic acid aqueous solution and 5 mM ammonium formate, and solvent B, consisting of 0.1% formic acid and 5 mM ammonium formate in acetonitrile	
	Column Type		Sepax GP-C18, (150 × 4.6 mm, 5 µm)	
	Gradient run program		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.	

Results and Discussion

Physical characterization of honey

Colour characteristics

Honey color is the primary characteristic for honey classification according to USDA-approved colour standards.[34] In the current study, the honey colours ranged from dark Amber for *Trigona* honey to white for citrus honey (Table 2, Figure 1). *Trigona* honey showed the highest Pfund value (135.5 mm Pfund). Pearson correlation showed a significant positive correlation between the colour of honey and the concentration of polyphenols and flavonoids as there was a proportional increase in the colour intensity concerning higher polyphenols and flavonoid content ($p<0.001$) (Table 4). The escalation in the colour intensity is significantly proportional to the antioxidant properties and phenolic content.[21] The Pfund value of *Trigona* honey is similar to sourwood honey [35] and some Bangladeshi honey.[36]

In nature, honey can be found in several colours, the variety ranges from light yellow to black in extreme cases, and in some occasions, green and red hues are present.[37] There are considerable reasons for the changes in the colour of honey as it is usually darkening with age.[4] Additionally, the ways of handling honeycombs, beekeeper's interventions as well as exposure to metals, sunlight, or high temperatures modulate the honey's colour.[38] For all these reasons, the colour of untreated honey is of crucial importance to commercial value and correlates significantly to the botanical origins.[4]

Moisture content and pH

The quality of honey can be distinguished by several factors among them is the moisture content. Low moisture content provides natural protection against fungal spoilage caused by the action of osmotolerant yeast fermentation during honey storage, which can convert carbohydrates into ethyl alcohols. Oxidized alcohol can be further converted to acetic acid and water, which gives the sour taste.[39] High moisture content is considered to be disadvantageous for long shelf life during honey storage.[40]

The moisture content of the current study for *Trigona*, *C. hyalolepis*, and Citrus honey was 25.79%, 17.29%, and 16.86%, respectively (Table 2). The values of the three honey samples were consistent with their relevant honey samples measured in other countries, for instance; *Trigona* honey moisture was similar to those of other stingless bee honey found in Thailand (25.27%-41.25%).[41] Additionally, Al-Mahasneh *et al*., (2013) has reported a percentage moisture content of 16.9% and 17.3% for *C. hyalolepis* and Citrus honey, respectively in Palestine. Likewise, moisture content for Moroccan honey was 14.3 to 20.2%[43] and 17.2-21.6% for Indian honey.[44]

The pH of the three types of honey was found to be acidic (Table 2), *Trigona* honey showed the highest acidity with pH 3.41 whereas the mean pH was 3.54 ± 0.12 for the three samples. The pH of honey is significantly dependent on the amount of amino acids and fatty acids secreted by the bees.[45] pH influences the honey texture, taste, and shelf life.[45] Sugars could be converted into hydroxyl methyl furfural (HMF) if overheated while processing or during long-term storage. This metabolic conversion can lower the total sugar content and endows a sour flavour.[44] The pH values were consistent with the pH readings reported in Malaysian, Indian, Bangladeshi, and Brazilian honey (between pH 3.49 and 4.70).[36,44,46]

Total Phenolic and flavonoid contents

There was a significant variation in the phenolic content among the three honey types as the total phenolic content ranged between 288.09 and 663.19 mg GAE/kg of honey (Table 2, Figure 2). *Trigona* honey encompasses the highest phenolic content (663.19 mg GAE/kg) followed by *C. hyalolepis* honey (471.87 mg GAE/kg), both of which were higher than Citrus honey (288.09 mg GAE/Kg). Likewise, *Trigona* honey contained the highest flavonoid content (237 mg Rut E/kg of honey followed by *C. hyalolepis* honey (168.3 mg Rut E/kg) and Citrus honey (70.62 mg Rut E/kg) (Table 2, Figure 2). The phenolic content of *Trigona* honey is higher than most of the known honey types as Bangladeshi honey ranged from 152.4 to 688.5 mgGAE/kg),[36] black mangrove honey (233.6 mgGAE/kg) and Christmas vine honey (213.9 mgGAE/kg),[47] Slovenian fir and forest honey at 241.4 and 233.9 mgGAE/kg,[48] and some Algerian honey (411.10 to 498.16 mgGAE/kg).[36] On the other hand, *Trigona* honey showed a significantly higher phenolic content in comparison with other known Malaysian honey such as Pineapple honey (277.5 mg GAE/kg),[49] and Tualang honey (251.7 ± 7.9 mgGAE/kg).[50] *Trigona* honey's high phenolic content is significantly attributed to the amounts of phenolic substances present in the tropical plant pollens. The content and amount of phenolic compounds differ thoroughly according to several factors among which are the season of collection, type of flowers, floral origins of honey, temperature, and humidity.[51]

Antioxidant activity

Free radicals scavenging capability of the three honey types were determined using a UV-visible spectrophotometer. The percentages of free radical scavenging activity were depicted in Table 2. The needed concentration to obtain a 50% reduction of DPPH free radicals (IC_{50} value) was used to express the antioxidant ability of each honey sample (Table 2, Figure 3). To determine the IC_{50} value for each

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

honey type, a dose-response curve was assessed using linear regression analysis. *Trigona* honey showed ultimately the highest ability for DPPH radical scavenging as the IC_{50} value was 61.042 ± 0.45 mg/mL whereas *C. hyalolepis* honey and Citrus Honey showed IC_{50} 0f 120.29 ± 1.64 mg/mL and 129.51 ± 4.3 mg/mL respectively. These values point to the presence of phenolic and flavonoid compounds in *Trigona* honey with high antioxidant potential.[25] Overall, in the current study, the antioxidant potential of Malaysian *Trigona* honey is

significantly higher than the Jordanian honey types (Table 2, Figure 4). Furthermore, comparative studies on honey showed high antioxidant activity of *Trigona* honey over other kinds, including Malaysian Gelam honey,[52] Algerian honey,[36] and Indian honey.[44] Statistical analysis has revealed a significant negative correlation between the IC_{50} values for the three honey samples and the proportional concentration of polyphenols (Table 4). *Trigona* honey showed the highest value ($r = -0.802$, $p < 0.05$) whereas *C. hyalolepis* honey and citrus honey showed ($r = -0.507$ and -0.451, $p < 0.05$), respectively. Table 2**:** The physicochemical properties for the three types of honey

Table 2: The physicochemical properties for the three types of honey

	Trigona H	*Centaurea hyalolepis* H	Citrus H
Colour	135.8 mm (Dark Amber)	39.29 mm (Extra light amber)	17.38 mm (White)
Moisture	25.79 ± 0.23	17.29 ± 0.61	16.86 ± 0.15
pH	3.41	3.66	3.54
Total sugars	55.4%	62.7%	62.6%
Fructose (g/100 g)	17.488 ± 0.144	15.915 ± 0.265	17.003 ± 0.435
Glucose (g/100 g)	36.951 ± 0.930	44.491 ± 0.484	43.7 ± 0.485
Sucrose (g/100 g)	0.97 ± 0.637	2.377 ± 0.372	1.911 ± 0.0352
Polyphenols content (mg GAE/Kg)	663.19 ± 19.54	471.87 ± 36.47	288.09 ± 1.31
Flavonoids content (mg Rut.E/Kg)	237.25 ± 8.025	168.33 ± 13.69	70.62 ± 8.50
Anti-oxidant activity (IC_{50} mg/mL)	61.042 ± 0.45	120.29 ± 1.64	129.51 ± 4.30

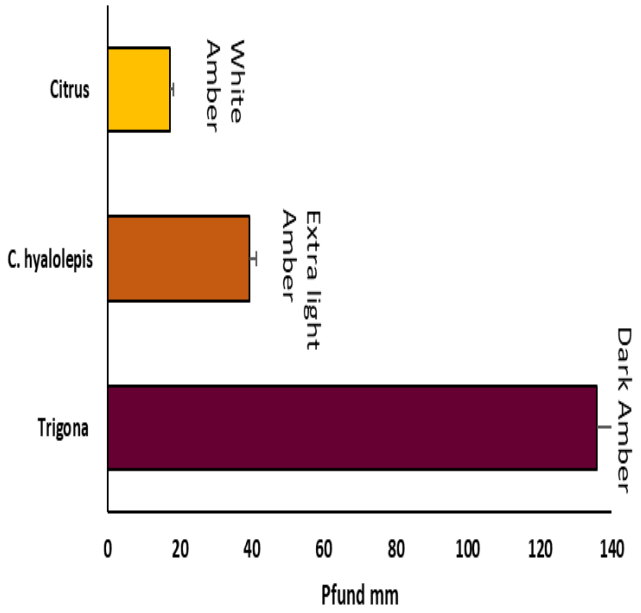

Figure 1: Colour characteristics of different honey samples. *Trigona* honey has the darkest colour among the three honey samples.

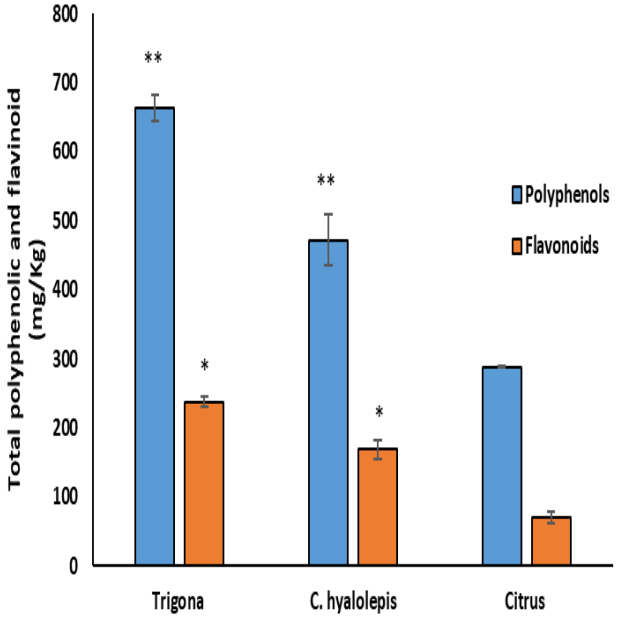

Figure 2: Total phenolic and flavonoids contents of honey. The highest content of polyphenols and flavonoids was demonstrated in *Trigona* honey. There was a significant difference in polyphenols and flavonoid concentration among the three honey samples. **= $P \leq 0.01$, *= $P \leq 0.05$

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

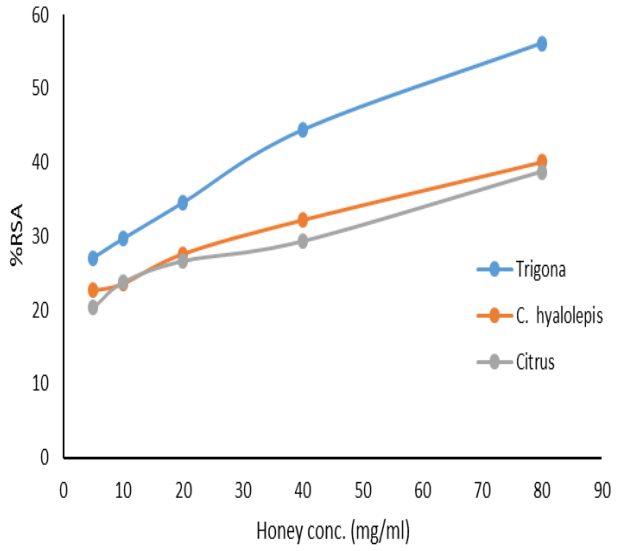

Figure 3: Percentage of radical scavenging activity (%RSA) at different concentrations. *Trigona* honey showed the highest antioxidant activity in comparison with *C. hyalolepis* and Citrus honey.

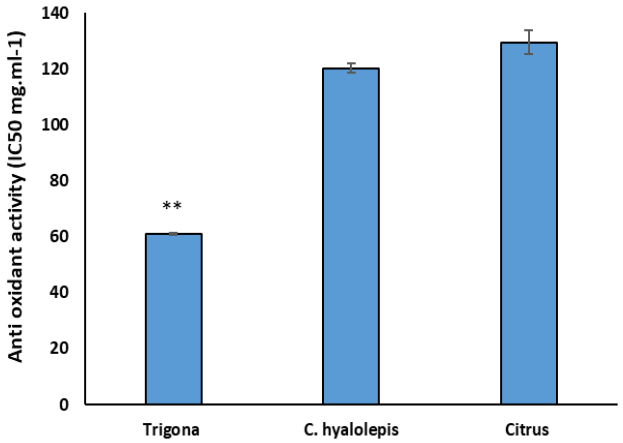

Figure 4: Antioxidant activity of different honey samples. *Trigona* honey showed the lowest IC_{50} value in comparison with *C. hyalolepis* and Citrus honey. **= $P \leq 0.01$.

Total sugar content

The total amount of sugar content of the three honey samples in the current investigation showed a range between 55.4% and 62.7% (Table 2). The total sugar content (55.4%) for *Trigona* honey was the lowest, whereas 62.7% and 62.6% were recorded for *Centaurea hyalolepis* and Citrus honey, respectively (Table 2). The results are consistent with the levels reported for stingless bee honey 54.90–87.00% in tropical regions,[53] while the proportions were 43.3 to 66.7% for *C. hyalolepis* and Citrus honey conducted in Palestine.[54]

Identification of phenolic profile by LC-MS/MS

Honey is constituted mainly from around 85% monosaccharide mostly fructose and glucose, while the remaining 15% is composed of water.[55] Furthermore, distinct phenolic compounds, small amounts of proteins, amino acids, vitamins, and enzymes were reported as variable ingredients of honey.[5] Characterization of *Trigona*, *C. hyalolepis*, and Citrus honey was carried out using mass spectrometry.

The analysis revealed more than 2000 metabolic compounds with different concentrations. Thirty (30) polyphenolic compounds that belong to phenols and flavonoids were identified. The identified compounds are presented in Table 3 and Figure 5). The highest concentration of the polyphenolic compound identified in *Trigona* was for quercetin with an intensity of up to 5.11E+06 count per second (CPS) whereas for *C. hyalolepis* honey, the intensity was 2.34E+06 (CPS) and for citrus honey 1.05E+06 (CPS) (Table 3, Figure 5). In the current study, we speculated to find a unique phenolic compound that harbors antioxidant activity presents only in *Trigona* honey when compared with *C. hyalolepis* and Citrus honey. Unfortunately, we could not identify a novel compound in *Trigona* honey not present in the other two honey samples. The rational reason for such activity of *Trigona* honey was explained by conducting a calculation for the amounts of polyphenols in *Trigona* and the intensity ratio for the highest 30 phenolic compounds reported in the current study; it was found a higher phenolic percentage of 42X times in *Trigona* over the Citrus honey whereas, there were 28X times over *Centaurea hyalolepis* honey. We speculate that this difference in the concentration of polyphenols endows a synergistic effect between the phytochemical compound present in *Trigona* honey over the other honey types used in the current study.

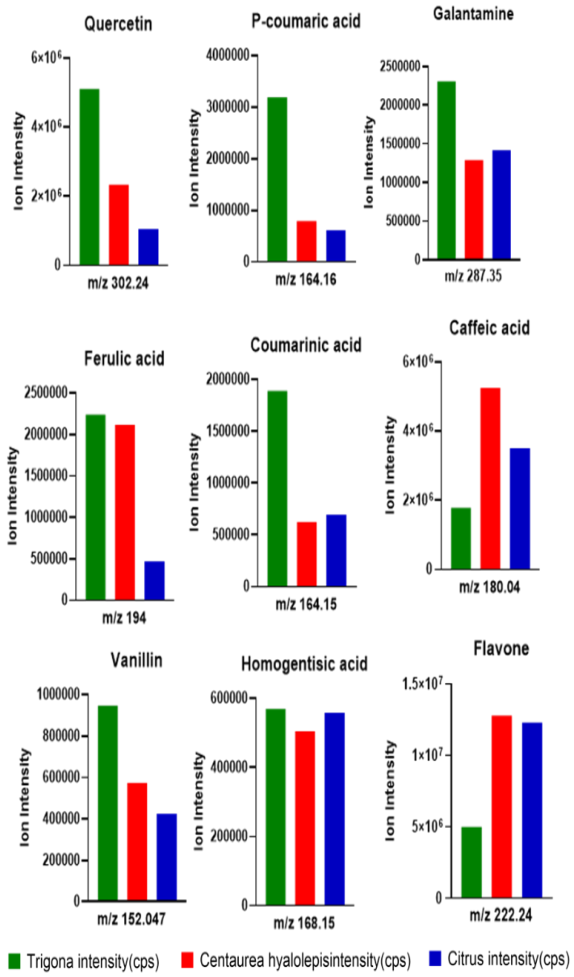

Figure 5: Ion intensity comparison for selected phytochemical compounds in the three honey samples

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 3: Comparison of Polyphenolic compounds identified using LC-MS/MS

Peak No	Compounds	Ion mode	[M ± H]- (Frag. MS2 m/z)	Molecular mass	*Trigona* intensity(cps) *Trigona* intensity(cps)	*Centaurea hyalolepis* intensity(cps)	Citrus intensity(cps)	*Trigona* (intensity ratio)	*C. hyalolepis* (intensity ratio)	Citrus (intensity ratio)
1	Pyrogallol	Negative	125.01 (69.1)	126.11	3.69E+05	4.93E+05	4.76E+05	0.78	1.04	1.00
2	4-Hydroxybenzoic acid	Negative	137 (93)	138.12	3.45E+05	6.36E+05	3.94E+05	0.88	1.61	1.00
3	3,4 -dihydroxy benzaldehyde	Negative	137 (91.93, 92.8, 95)	138.12	5.50E+05	3.36E+05	5.56E+05	0.99	0.60	1.00
4	Cinnamic acid	Negative	147 (102, 118, 129)	148.05	2.94E+05	4.65E+05	3.66E+05	0.80	1.27	1.00
5	Vanillin	Negative	150.95 (135.9)	152.05	9.46E+05	5.73E+05	4.23E+05	2.24	1.35	1.00
6	Protocatechuic Acid	Negative	153(81, 91.02, 108)	154.03	2.95E+05	7.03E+05	3.08E+05	0.96	2.28	1.00
7	Coumarinic acid	Negative	163.01 (119.04)	164.15	1.89E+06	6.25E+05	6.94E+05	2.72	0.90	1.00
8	P-coumaric acid	Negative	163 (119)	164.16	3.19E+06	7.94E+05	6.01E+05	5.31	1.32	1.00
9	Homogentisic acid	Negative	167.03 (123.03)	168.15	5.70E+05	5.05E+05	5.58E+05	1.02	0.91	1.00
10	*vanillic acid*	Negative	166.98 (151.97)	168.15	4.86E+05	4.95E+05	4.50E+05	1.08	1.10	1.00
11	Gallic acid	Negative	169 (125)	168.15	3.13E+05	3.95E+05	7.82E+05	0.40	0.51	1.00
12	Caffeic acid	Negative	179 (135)	180.04	1.78E+06	5.26E+06	3.50E+06	0.51	1.50	1.00
13	Ferulic acid	Negative	193.03 (134)	194	2.24E+06	2.12E+06	4.62E+05	4.85	4.59	1.00
14	Syringic acid	Negative	197 (182, 147)	198.05	4.24E+05	2.76E+05	7.32E+05	0.58	0.38	1.00
15	Flavone	Negative	221 (193)	222.24	5.01E+06	1.28E+07	1.23E+07	0.41	1.04	1.00
16	Chrysin	Negative	253 (209, 178)	254.06	4.86E+05	7.10E+05	6.45E+05	0.75	1.10	1.00
17	Genistein	Negative	269 (215, 143)	270.24	3.72E+05	6.22E+05	2.00E+05	1.86	3.11	1.00
18	Apigenin	Negative	269 (251, 269)	270.25	4.04E+05	1.50E+05	2.84E+05	1.42	0.53	1.00
19	Naringenin	Negative	271 (107, 119)	272.07	1.20E+05	1.12E+05	4.97E+04	2.41	2.25	1.00
20	Caffeic acid phenethyl ester	Negative	283.6 (241, 221, 179)	284.31	2.51E+05	1.27E+05	3.38E+05	0.74	0.38	1.00
21	Kaempferol	Negative	285 (133, 151, 175)	286.23	1.34E+05	1.27E+05	1.11E+05	1.21	1.14	1.00
22	Luteolin	Negative	284.91 (107	286.24	1.13E+05	1.17E+05	5.45E+04	2.07	2.15	1.00
23	Galantamine	Positive	288.1 (198)	287.35	2.31E+06	1.29E+06	1.41E+06	1.64	0.91	1.00
24	Catechine	Negative	289 (271)	290.08	1.60E+05	2.65E+04	2.26E+05	0.71	0.12	1.00
25	Quercetin	Positive	303 (137)	302.24	5.11E+06	2.34E+06	1.05E+06	4.87	2.23	1.00
26	Hesperetin	Negative	301.02 (135)	302.28	1.82E+05	7.94E+04	4.20E+04	4.33	1.89	1.00
27	Myricetin	Negative	316.9 (107)	318.23	1.22E+05	4.90E+04	1.52E+04	8.03	3.22	1.00
28	Catechin gallate	Negative	441 (168.8)	442.4	3.49E+05	6.19E+04	2.19E+04	15.94	2.83	1.00
29	Rutin	Negative	609(301)	610.52	3.63E+04	2.27E+04	3.02E+04	1.20	0.75	1.00
30	Catechin 3',5-diglucoside	Negative	614 (452)	614.5	4.10E+04	4.20E+04	4.00E+04	1.03	1.05	1.00

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 4: Correlation between physicochemical properties, polyphenolic, and flavonoid content, and the antioxidant activity of the three honey types

		DPPH	**Polyphenols**	**Flavonoids**	**pH**	**Color**
		Pearson Correlation	Pearson Correlation	Pearson Correlation	Pearson Correlation	Pearson Correlation
DPPH	*Trigona*	1	**-.820***	**-.812***	**-.861***	**-.813***
	C. hyalolepis	1	**-0.507***	**-0.435***	0.508	**-0.493***
	Citrus	1	**-0.451***	**-0.467***	**-0.487**	**-0.519***
Polyphenols	*Trigona*		1	**.969****	**.978****	**.986****
	C. hyalolepis		1	**.997****	**0.485***	**0.827****
	Citrus		1	0.729	**.937****	**0.767****
Flavonoids	*Trigona*			1	**.974****	**.985****
	C. hyalolepis			1	0.554	**.794****
	Citrus			1	0.444	**.713****
pH	*Trigona*				1	**.993****
	C. hyalolepis				1	0.499
	Citrus				1	0.494
Color	*Trigona*					1
	C. hyalolepis					1
	Citrus					1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Conclusion:

To the best of our knowledge, this is the first study that investigated the phytochemical characterization of two different types of honey from two different geographical regions; Malaysian stingless bee honey (*Trigona*) versus Jordanian honey using LC-MS/MS.
Malaysian *Trigona* honey has a higher antioxidant activity over the other types of honey, which was attributed to the high concentration of total polyphenolic compounds in its composition. It is highly recommended to add *Trigona* honey as a fortification ingredient in food products because of the valuable nutritional and therapeutic values against oxidative stress-related diseases.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

We are much thankful to the medical campus of UniSZA (Universiti Sultan Zainal Abidin) for funding this work through the award of the grant; Dana Penyelidikan UniSZA 2018/DPU/13 R0034-R013. Also, we thank the Deanship of Scientific Research at the University of Petra and The University of Petra Pharmaceutical Center (UPPC) for their cooperation in providing the infrastructure to conduct this research.

References

1. Jibril FI, Hilmi ABM, Manivannan L. Isolation and characterization of polyphenols in natural honey for the treatment of human diseases. Bull Natl Res Cent. 2019;43(1):4-11
2. Aw Yong PY, Islam F, Harith HH, Israf DA, Tan JW, Tham CL. The Potential use of Honey as a Remedy for Allergic Diseases: A Mini Review. Front Pharmacol. 2021;11(1):2061-2070.
3. Al-kafaween MA, Hilmi ABM, Jaffar N, Al-Jamal HAN, Zahri MK, Jibril FI. Antibacterial and Antibiofilm activities of Malaysian Trigona honey against Pseudomonas aeruginosa ATCC 10145 and Streptococcus pyogenes ATCC 19615. Jordan J Biol Sci. 2020;13(1):69-76
4. Al-Farsi M, Al-Amri A, Al-Hadhrami A, Al-Belushi S. Color, flavonoids, phenolics and antioxidants of Omani honey. Heliyon. 2018;4(10):874-888
5. Ramanauskiene K, Stelmakiene A, Briedis V, Ivanauskas L, Jakštas V. The quantitative analysis of biologically active compounds in Lithuanian honey. Food Chem. 2012;132(3):1544-1548
6. Rao PV, Krishnan KT, Salleh N, Gan SH. Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. Rev Bras Farmacogn. 2016;26(5):657-664
7. Liyanage D, Mawatha B. Health benefits and traditional uses of honey: A review. J Apith. 2017; 2(1):9-14.
8. Kuropatnicki AK, Kłósek M, Kucharzewski M. Honey as medicine: historical perspectives. J Apic Res. 2018; 57(1):113-118.
9. Al-Kafaween MA, Al-Jamal HAN, Hilmi ABM, Elsahoryi NA, Jaffar N, Zahri MK. Antibacterial properties of selected Malaysian Tualang honey against Pseudomonas aeruginosa and Streptococcus pyogenes. Iran J Microbiol. 2020; 12(6):565-576.
10. Khleifat KM, Qaralleh H, Al-limoun MO, Al-khlifeh EM, Aladaileh SA, Tawarah N, Almajali IS. Antibacterial and Antioxidant Activities of Local Honey from Jordan. Trop J Nat Prod Res. 2021;5 (3):470-477.

11. Rayyan WA, Alshammari SA, ALSammary AM, AL-Shammari MS, Seder N, Abu-Qatouseh LF. The Phytochemical Analysis and Antimicrobial Activity of Pergularia Tomentosa in North East Kingdom of Saudi Arabia KSA. Biomed Pharmacol. 2018;11(4):1763-1771.
12. Ahmed S and Othman NH. Honey as a potential natural anticancer agent: a review of its mechanisms. Evid-Based Compl Altern Med. 2013;2013:1-7.
13. Jull AB, Cullum N, Dumville JC, Westby MJ, Deshpande S, Walker N. Honey as a topical treatment for wounds. Cochrane Database Syst Rev. 2015;1(3):256-259.
14. Abd El-Malek FF, Yousef AS, El-Assar SA. Hydrogel film loaded with new formula from manuka honey for treatment of chronic wound infections. J Glob Antimicrob. 2017; 11:171-176.
15. Al-Nadaf AH, Seder NJ, Rayyan WA. Wound healing; antimicrobial and anti-oxidant activity for Jordanian *Juglans Regia* L. unripe fruits. J Innov Pharm. 2018;5(3):26-34.
16. Hoover SE and Ovinge LP. Pollen collection, honey production, and pollination services: managing honey bees in an agricultural setting. J. Econ. Entomol. 2018;111(4):1509-1516
17. Brosi BJ. The complex responses of social stingless bees (Apidae: Meliponini) to tropical deforestation. For Ecol. 2009; 258(9):1830-1837
18. Aksoy A, Ertürk YE, Erdogan S, Eyduran E, Tariq MM. Estimation of honey production in beekeeping enterprises from eastern part of Turkey through some data mining algorithms. Pak J Zool. 2018; 50(6):2199-2207.
19. Al-Ghamdi AA, Adgaba N, Herab AH, Ansari MJ. Comparative analysis of profitability of honey production using traditional and box hives. Saudi J Biol Sci. 2017; 24(5):1075-1080.
20. Erejuwa OO, Sulaiman SA, Ab Wahab MS. Honey: a novel antioxidant. Molecules. 2012; 17(4):4400-4423.
21. Dżugan M, Tomczyk M, Sowa P, Grabek-Lejko D. Antioxidant activity as biomarker of honey variety. Molecules. 2018; 23(8):2069-2077.
22. Stagos D, Soulitsiotis N, Tsadila C, Papaeconomou S, Arvanitis C, Ntontos A, Karkanta F, Adamou‐Androulaki S, Petrotos K, Spandidos DA. Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. Int J Mol Med. 2018; 42(2):726-734.
23. Avila S, Beux MR, Ribani RH, Zambiazi RC. Stingless bee honey: Quality parameters, bioactive compounds, health-promotion properties and modification detection strategies. Trends Food Sci. Technol. 2018; 81:37-50.
24. Zafar M, Latafat T, Zehra A, Farooqui Y. Therapeutic Properties of Honey: A Review of Literature. Res Rev A J Pharmacol. 2020;10(1):41-49.
25. Ng WJ, Chan YJ, Lau ZK, Lye PY, Ee KY. Antioxidant properties and inhibitory effects of trigona honey against Staphylococcus aureus planktonic and biofilm cultures. Int J. 2017; 12(37):28-33.
26. Ferreira IC, Aires E, Barreira JC, Estevinho LM. Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract. Food Chem. 2009; 114(4):1438-1443.
27. da Silva PM, Gauche C, Gonzaga LV, Costa ACO, Fett R. Honey: Chemical composition, stability and authenticity. Food Chem. 2016;196:309-323.
28. Helrich K. Official methods of analysis, vols. 1 and 2. (15th) ed. Arlington, VA: The Association; 1990. 64-85 p.
29. Ali F, Ranneh Y, Ismail A, Esa NM. Identification of phenolic compounds in polyphenols-rich extract of Malaysian cocoa powder using the HPLC-UV-ESI—MS/MS and probing their antioxidant properties. J Food ScinTechnol. 2015; 52(4):2103-2111.
30. Liu S-C, Lin J-T, Wang C-K, Chen H-Y, Yang D-J. Antioxidant properties of various solvent extracts from lychee (Litchi chinenesis Sonn.) flowers. Food Chem. 2009; 114(2):577-581.
31. Chang C-C, Yang M-H, Wen H-M, Chern J-C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 2002;10(3):8-14.
32. Isla MI, Craig A, Ordoñez R, Zampini C, Sayago J, Bedascarrasbure E, Alvarez A, Salomón V, Maldonado L. Physico chemical and bioactive properties of honeys from Northwestern Argentina. LWT-Food Sci Technol. 2011;44(9):1922-1930.
33. Chua LS, Rahaman NLA, Adnan NA, Eddie Tan TT. Antioxidant activity of three honey samples in relation with their biochemical components. J Anal Methods Chem. 2013;2013:1-8.
34. USDA. Extracted Honey Grades and Standards, 1985. Accessed 4 May 2021 [Available from: https://www.ams.usda.gov/grades-standards/extracted-honey-grades-and-standards.
35. Moniruzzaman M, Sulaiman SA, Khalil MI, Gan SH. Evaluation of physicochemical and antioxidant properties of sourwood and other Malaysian honeys: a comparison with manuka honey. Chem Cent J. 2013;7(1):1-12.
36. Islam A, Khalil I, Islam N, Moniruzzaman M, Mottalib A, Sulaiman SA, Gan SH. Physicochemical and antioxidant properties of Bangladeshi honeys stored for more than one year. BMC Compl Altern Med. 2012; 12(1):177-186.
37. Selvaraju K, Vikram P, Soon JM, Krishnan KT, Mohammed A. Melissopalynological, physicochemical and antioxidant properties of honey from West Coast of Malaysia. J Food Sci Technol. 2019; 56(5):2508-2521.
38. Madhu G, Kumar AS, Nair SK, editors. Sunlight-induced honey-mediated green synthesis of silver nanoparticles. AIP Conference Proceedings; AIP Publishing LLC. 2019.
39. Chirife J, Zamora MC, Motto A. The correlation between water activity and% moisture in honey: Fundamental aspects and application to Argentine honeys. J Food Eng. 2006;72(3):287-292.
40. Singh I and Singh S. Honey moisture reduction and its quality. J Food Sci Technol. 2018; 55(10):3861-3871.
41. Chuttong B, Chanbang Y, Sringarm K, Burgett M. Physicochemical profiles of stingless bee (Apidae: Meliponini) honey from South east Asia (Thailand). Food Chem. 2016; 192:149-155.
42. Al-Mahasneh M, Rababah T, Ma'Abreh A. Evaluating the Combined Effect of Temperature, Shear Rate and Water Content on Wild-Flower Honey Viscosity Using Adaptive Neural Fuzzy Inference System and Artificial Neural Networks. J Food Proc Eng. 2013; 36(4):510-520.
43. Chakir A, Romane A, Marcazzan GL, Ferrazzi P. Physicochemical properties of some honeys produced from different plants in Morocco. Arab J Chem. 2016; 9:S946-S954.
44. Saxena S, Gautam S, Sharma A. Physical, biochemical and antioxidant properties of some Indian honeys. Food Chem. 2010;118(2):391-397.
45. Mureșan CI, Buttstedt A. pH-dependent stability of honey bee (Apis mellifera) major royal jelly proteins. Sci Rep. 2019; 9(1):1-6.
46. Mateo R and Bosch-Reig F. Classification of Spanish unifloral honeys by discriminant analysis of electrical conductivity, color, water content, sugars, and pH. J Agric Food Chem. 1998; 46(2):393-400.
47. Alvarez-Suarez JM, Tulipani S, Díaz D, Estevez Y, Romandini S, Giampieri F, Damiani E, Astolfi P, Bompadre S, Battino M. Antioxidant and antimicrobial capacity of several monofloral Cuban honeys and their correlation with color, polyphenol content and other chemical compounds. Food Chem Toxicol. 2010; 48(8-9):2490-2499.
48. Bertoncelj J, Doberšek U, Jamnik M, Golob T. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. Food Chem. 2007; 105(2):822-828.

49. Sari DYI, Wijayanti HS, Afifah DN. Total phenolic content, antioxidant activity and organoleptic test of Nata de pina between various parts of honey pineapple variety (*Ananas comosus* [L.] Merr. Var. Queen). J Food Res. 2021; 5(4):169-174.
50. Mohamed M, Sirajudeen K, Swamy M, Yaacob M, Sulaiman S. Studies on the antioxidant properties of Tualang honey of Malaysia. Afr J Trad Compl Altern Med. 2010;7(1):59-63.
51. MartíNez RA, Schvezov N, Brumovsky LA, Román ABP. Influence of temperature and packaging type on quality parameters and antimicrobial properties during Yateí honey storage. J Food Sci Technol. 2017; 38:196-202.
52. Khalil MI, Mahaneem M, Jamalullail SMS, Alam N, Sulaiman SA. Evaluation of radical scavenging activity and colour intensity of nine Malaysian honeys of different origin. J ApiProd ApiMed Sci. 2011;3(1):04-11.
53. Zulkhairi Amin FA, Sabri S, Mohammad SM, Ismail M, Chan KW, Ismail N, Norhaizan ME, Zawawi N. Therapeutic properties of stingless bee honey in comparison with european bee honey. Adv Pharm Pharm Sci. 2018; 2018:1-12.
54. Abdulkhaliq A and Swaileh KM. Physico-chemical properties of multi-floral honey from the West Bank, Palestine. Int J Food Prop. 2017;20(2):447-454.
55. Santos-Buelga C and González-Paramás AM. Chemical composition of honey. Bee Products-Chemical and Biological Properties: Springer; 2017. 43-82 p.