

## Phytochemical content, antioxidant activity and mineral elements of honey produced by four different species of Malaysian stingless bees

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### Abstract

This study investigated the phytochemical composition of honey from four species of Malaysian stingless bee i.e. *Tetragonula laeviceps*, *Geniotrigona thoracica*, *Lepidotrigona terminata*, as well as *Heterotrigona itama*. The measured phytochemical composition was total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, pH, colour intensity, and mineral element analysis i.e. Magnesium (Mg), Calcium (Ca), Zinc (Zn), Potassium (K), and Sodium (Na). The production of honey by the *L. terminata* had the highest TPC value (6.74±0.57 mg GAE/100 g FW) while the *T. laeviceps* possesses the highest TFC value (14.85±3.71 mg QE/100 g FW). In the antioxidant activity measurement, honey from the *H. itama* obtained the highest percentage of 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibition (29.52±0.45%) and honey from *T. laeviceps* displayed the highest value in the Ferric reducing antioxidant power (FRAP) analysis (0.95±0.013 abs). The four distinct stingless bee species' colour intensity demonstrated the variation of colour intensity in the range of 0.09-0.23 abs mAU. All honey obtained the pH values (4.75-5.03) within the acidic medium. Mg is the major mineral element in all honey samples which is followed by Na, Ca, K, and Zn.

## 1. Introduction

Honey is a supersaturated sugar solution containing beneficial natural remedy that is popular for food and medicinal usage. It comes from the plant's floral nectar part which is being converted into honey using the enzymes produced by the bees (Cantarelli *et al.*, 2008). Da Silva *et al.* (2013) reported that honey contains 80-85% carbohydrates, 15-17% water, 0.3% protein, and 0.2% ashes. The authors added that honey contains high fructose and glucose concentration, and low amino acids, minerals, organic acids, phenolic acids, vitamins, enzymes, and other phytochemicals levels.

Recently, studies on stingless bee honey gained wide attention in Malaysia. Stingless bee honey is sugary liquid, superb in taste, and odour (Chuttong *et al.*, 2016). Biluca *et al.* (2014) explained that the stingless bee

honey has various phytochemical content as opposed to regular honey bee in terms of colour, taste, water, viscosity, and sugar contents. Moreover, stingless bee honey has a greater content of polyphenols and flavonoids as compared to the honey that is produced by *Apis* spp. (Rodriguez-Malaver *et al.*, 2009; Rodriguez-Malaver *et al.*, 2013; Biluca *et al.*, 2016). In addition, Maringgal *et al.* (2019) indicated that stingless bee honey from six regions in Malaysia has significant variation in its phytochemical content in terms of total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity, colour intensity, and pH. The authors also found that the phytochemical content has a significant correlation with the botanical and geographical regions in Malaysia. A seminal study in this area is the work of Lim *et al.* (2019) who explained that the stingless bee honey's nutritional value is also

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affected by environmental, storage condition, and processing factors. Due to the excellent phytochemical content and the nutritional benefits, this honey was valued by consumers globally, that renders it commercially more valuable (Da Silva *et al.*, 2013). This also explained that the stingless bee honey's selling price can go up to \$100/ kg, which is almost twice the price of the honey bee (\$20-40/kg) (Shadan *et al.*, 2018).

Honey is also rich with mineral elements which represent its therapeutic value. Plant uptake from the soil and the environment from where the bees gather the nectar in the process of producing honey determine the mineral elements in it (Alves *et al.*, 2013; Alqarni *et al.*, 2014). Kek *et al.* (2017) reported that Malaysian raw honey contains high elements of potassium, sodium, and calcium. The authors added that the mineral element is higher for honey harvested from the forest or jungle when compared with honey that collected from farm plantation. Although there are 32 species of stingless bee that had been documented in Malaysia (Norowi *et al.*, 2010), studies on phytochemical content on the different stingless bee honey are still limited. This is supported by Kelly *et al.* (2014) who figure out that 17-32 stingless bee species are established in Malaysia, but only two species (*H. itama* and *G. thoracica*) are largely being used in the meliponiculture and research studies. The knowledge on composition as well as mineral elements of stingless bee honey from Malaysia is also hardly found. The quality of stingless bee honey differed from the standards that are laid out by the international honey standards in terms of its composition (Codex Alimentarius Commission, 2001). Thus, the present study is carried out with the following objectives: 1) to determine the phytochemical content consist of TFC, TPC, colour intensity, antioxidant activity, and pH of four honey samples produced by four different species of Malaysian stingless bee; 2) to determine the mineral elements of honey produced by four different species of Malaysian stingless bee.

## 2. Materials and methods

### 2.1 Preparation of Malaysia stingless bee honey samples

One litre of honey samples from four different species (*T. laeviceps*, *G. thoracica*, *L. terminata*, and *H. itama*) of stingless bee was collected from the Stingless Bee Farm, Universiti Putra Malaysia during December 2017 to March 2018. All the samples were harvested from the stingless beehives located in multibotany nectar source. All the samples were placed at room temperature in sterile airtight glass bottles in order to prevent moisture absorption during the sampling duration, analytical test, and storage. For the purpose of assessing the total phenolic content (TPC), total flavonoid content

(TFC), pH values, colour intensity, and antioxidant capacity i.e. 1,1-diphenyl-2-picrylhydrazyl (DPPH) inhibition and Ferric reducing antioxidant power (FRAP) activities, approximately two grams of honey were diluted in 20 mL of distilled water whereby it produces the concentration of 0.1 g/mL. Meanwhile, for the mineral elements analysis, 0.5 g of honey samples was kept in a Tetrafluoromethaxil (TFM) vessel with 2 mL of hydrogen peroxide (30% v/v) and 3 mL of nitric acid (65% v/v). Each analysis was repeated four times.

### 2.2 Phytochemical analysis

TPC and TFC of honey were analysed using the aluminum chloride colorimetric technique (Ali *et al.*, 2015) and the Folin-Ciocalteu reagent (Berretta *et al.*, 2005). About 200  $\mu$ L of honey solution was added to 3 mL of 10% diluted Folin-Ciocalteu reagent. The absorbance was computed at 750 nm with the use of a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510) after placing the solution in the dark at room temperature. The TPC value was stated as mg gallic acid equivalents per 100 g of honey.

To measure TFC, 2% of aluminium chloride solution was mixed with 1 mL of the honey sample (0.1-0.4 g/mL). The mixture's absorbance was calculated at 430 nm using a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510) after incubating it for 10 mins at 25°C. Based on the quercetin standard, with the use of a calibration curve, TFC was expressed in mg QUE/ 100 g FW.

#### 2.2.1 Measurements of antioxidant activity

##### 2.2.1.1 Inhibition of DPPH

A specific sample's scavenging activity is measured in electron transfer, which is the principal of DPPH method (Garcia *et al.*, 2012). Without direct exposure to light and at room temperature, the assay produces a constant concentrated violet solution. The honey's radical scavenging activity was determined via the use of Multiskan GO microplate spectrophotometer at 517 nm against the DPPH radical, as detailed out Beretta *et al.* (2005), in this research. The DPPH solution was prepared by dissolving 2 mg of DPPH in 100 mL of ethanol. Following that, 2 mL of DPPH solution was mixed with 1 mL of ethanolic honey solution. The reaction mixture was vigorously shaken to yield a good mixture and then stored for half an hour at the room temperature without exposing it to light. Following that, the mixture's absorbance was recorded. The honey's scavenging activity through DPPH radical was calculated from the equation below:

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

Where Abs control refers to the DPPH radical and ethanol absorbance, and Abs sample refers to the DPPH radical, and honey or ascorbic acid absorbance. The experiment was carried out four times.

### 2.2.1.2 FRAP analysis

FRAP is a parameter that measures the reductant or antioxidant activity of a sample. The reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  indicates the total reduction (Yen and Duh, 1993; Ahmad and Abdullah, 2013). In this research, the FRAP analysis was carried out by adding 0.5 g honey and 100 mL distilled water into 2.5 mL of phosphate buffer (0.2M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixtures were incubated for a total of 20 mins under 50°C. After that, 2.5 mL of 10% trichloroacetic acid was added to the mixture 0.5 mL 0.1% ferric chloride and 1 mL distilled water were added to the 1 mL mixture. The rate at which the solution was absorbed was calculated at 700 nm through the use of a Multiskan GO microplate spectrophotometer (Thermo Scientific 1510). The honey's FRAP activity was expressed in absorbance unit.

### 2.2.2 Measurements of pH and colour intensity

The colour intensity representing honey's darkness value was measured using the method detailed out in the previous research (Beretta et al., 2005). With the use of warm water at 45 to 50°C, a 50% (w/v) honey solution was prepared. The net absorbance was obtained from the use of Multiskan GO microplate spectrophotometer at 450 nm. The pH was measured with the use of a pH meter, model LAQUA twin pH (HORIVA, Japan).

### 2.2.3 Measurements of mineral elements

The mineral elements analysis was determined by using the method described by Kek et al. (2017). The TFM vessels with honey solutions were closed and later placed in the polypropylene rotor body, whereby this was later placed into a microwave digester (MLS 1200 Mega, Milestone S.r.l., Sorisole, Bergamo, Italy) for the digestion process to take place. After digestion, the rotor body's temperature was brought down to room

temperature. The sample digested was gathered and later diluted to 50 mL using deionised water for mineral analysis. Mineral elements of Magnesium (Mg), Sodium (Na), Calcium (Ca), Potassium (K), and Zinc (Zn) were determined with the use of the atomic absorption spectrometer (AAS) (GBC-906, GBC Scientific Equipment Pty. Ltd., Melbourne, Australia).

### 2.3 Statistical analysis

The analysis of significance between mean values were carried out using the one-way analysis of variance (ANOVA) and Tukey's multiple-comparisons analysis using SAS 9.4 software. The significant level was set to be  $P \leq 0.05$ .

## 3. Results and discussion

### 3.1 Total phenolic content (TPC) and total flavonoid content (TFC)

The TPC as well as the TFC values of Malaysian stingless bee honey samples are depicted in Table 1. The outcomes revealed that the TPC and TFC values have significant difference ( $P \leq 0.05$ ) among the species of stingless bee honey. The content of TPC found in honey samples ranged from 3.39 to 6.74 mg GAE/100 g FW. Samples from the *G. thoracica* honey had the lowest values of TPC, while honey from the *L. terminata* showed the highest value. The analysis also indicated that the TPC value of honey from *T. laeviceps* (5.52 mg GAE/100 g) is significantly different with *G. thoracica* value of 3.39 mg GAE/100 g, but insignificantly different from *L. terminata* (6.74 mg GAE/100 g) and *H. itama* (4.47 mg GAE/100 g).

The mean TFC constituent values of honey in four different ranged from 3.74 to 14.85 mg QUE/100 g FW. Honey from the *T. laeviceps* demonstrated the highest TFC followed by the *H. itama*, *L. terminata*, and *G. thoracica*. The one-way ANOVA also showed the same trend with TPC value whereby the TFC of honey from *T. laeviceps* with 14.85 mg QUE/100 g FW is statistically significantly different with *G. thoracica* with the value of 3.74 mg QUE/100 g FW, but no significant difference

Table 1. The TPC, TFC, antioxidant activities, colour intensity and pH in stingless bee honey from various species

Species	TPC (mg GAE/100 g FW)	TFC (mg QUE/100 g FW)	DPPH (%)	FRAP (abs)	Colour Intensity (abs, mAU)	pH
<i>T. laeviceps</i>	5.52±0.56 <sup>ab*</sup>	14.85±3.71 <sup>a</sup>	17.22±1.15 <sup>b</sup>	0.95±0.013 <sup>a</sup>	0.23±0.001 <sup>a</sup>	4.75±0.45 <sup>a</sup>
<i>G. thoracica</i>	3.39±0.06 <sup>c</sup>	3.74±0.07 <sup>b</sup>	12.34±0.95 <sup>c</sup>	0.91±0.01 <sup>b</sup>	0.09±0.001 <sup>c</sup>	5.03±0.47 <sup>a</sup>
<i>L. terminata</i>	6.74±0.57 <sup>a</sup>	9.98±0.71 <sup>ab</sup>	28.36±0.66 <sup>a</sup>	0.91±0.01 <sup>ab</sup>	0.18±0.01 <sup>b</sup>	4.95±0.53 <sup>a</sup>
<i>H. itama</i>	4.47±0.10 <sup>bc</sup>	13.05±0.23 <sup>a</sup>	29.52±0.45 <sup>a</sup>	0.898±0.001 <sup>b</sup>	0.23±0.002 <sup>a</sup>	4.85±0.44 <sup>a</sup>

Values are expressed as mean±SD. Values with the same superscript letters within the same column are not significantly different at  $P \leq 0.05$  by using Tukey test.

with *L. terminata* (9.98 mg QUE/100 g FW) and *H. itama* (13.05 mg QUE/100 g FW).

The different species of stingless bee honey could offer an explanation for the variation in TPC value. Similar researches were performed by Biluca et al. (2016) describing the TPC values from 10.3 to 98.0 mg GAE/100 g for ten different species of stingless bee honey from Santa Catarina, Brazil. Seminal contributions have been made by Silva et al. (2013) and Da Silva et al. (2013) found that the TPC values ranged from 1.30 to 66.00 mg GAE/100 g for stingless bee honeys from Amazon and Paraiba, Brazil. The current study also showed that the TFC of honey differ significantly between different stingless bee species. On the other hand, a study by Fadzilah et al. (2017) discovered the TFC of honey values ranged from 15.28 to 31.80 mg/g QE for three different stingless bee species in Malaysia; *T. apicalis*, *T. itama*, and *T. thoracica*.

Apart from different species, the TPC and TFC values of stingless bee honey are also affected by the geographical and botanical origins of the nectar gathered in producing the honey. Recently, Maringgal et al. (2019) revealed the TPC and TFC values of Malaysian stingless bee honey varied significantly among the geographical locations in Malaysia. These findings corroborate with the current studies by Majid et al. (2019), who showed the values of TPC and TFC of stingless bee honey produced by *H. itama* were influenced by the geographical floral origins in Johor, Malaysia.

### 3.2 Antioxidant activity

The honey samples' DPPH radical scavenging effect is depicted in Table 1. Stingless bee honey from *H. itama* had the greatest ability to scavenge DPPH radicals with the DPPH inhibition by 29.52% ( $P \leq 0.05$ ) followed by *L. terminata*, and *T. laeviceps*. Meanwhile, *G. thoracica* demonstrated the lowest value in DPPH inhibition of 12.34%. The current study also indicated that the DPPH inhibition values showed statistically significant difference among the stingless bee species. However, there was no significant difference found in the DPPH inhibition values between honey that produced *L. terminata* and *H. itama*.

The finding seems to be consistent with Tuksitha et al. (2018) who found that DPPH assay content of the three different honeys produced by three stingless bees species (*G. thoracica*, *H. itama*, and *H. erythrogastra*) from Sarawak (Eastern of Malaysia) are ranging from 17.0±7.5% to 47.4±3.2%. Whereas, Abu Bakar et al. (2017) found that *H. itama* honey collected in Melaka (Western of Malaysia) showed the highest DPPH assay

level (97.30±0.84%). However, Mohd Nasir et al. (2019) explained in contradict way where honey collected in Besut and Dungun, Terengganu that produced by *H. itama*, *T. laeviceps*, and *G. thoracica* species does not possess good antioxidant activity with the DPPH inhibition value were below than 50%.

The values of DPPH inhibition in honey are always carried out a large variety of amount since the values depend on the botanical and geographical origin as well as the bee species (Boussaid et al., 2014; Habib et al., 2014). The difference in the species of stingless bee may explain the variations of DPPH inhibition values in current studies, even though the honey was collected at the same geographical origin. It was supported by Agus et al. (2019) who found that different geographical origins of meliponiculture influenced the antioxidant activity of honey produced by *T. laeviceps* species. The authors also added that antioxidant activity DPPH of *T. laeviceps* honey had a significant effect on TPC and TFC.

The average values of FRAP analysis (Table 1) of honey from the *H. itama* demonstrated the lowest antioxidant potential while the honey produced by *T. laeviceps* obtained the highest values ( $P \leq 0.05$ ), depicting the highest reducing power of  $Fe^{3+}$ . Surprisingly, no significant differences were found in the FRAP values of honey among the stingless bee species. However, the FRAP values of honey produced by *T. laeviceps* and *G. thoracica* have significant differences. These outcomes agree with those of Biluca et al. (2016) who found that different species of stingless bee showed significant differences in reducing the power of  $Fe^{3+}$ . Moreover, the authors found that the honey sample produced by *M. marginata* species resulted in the utmost reducing power of  $Fe^{3+}$  (624 mmol Fe II/100 g) and the lowest reducing power is found in the honey sample harvested from the *M. bicolor* species (61.1 mmol Fe II/100 g).

### 3.3 pH and colour

Normally, stingless bee honey has sourness in taste and a distinct aroma due to their acidic nature. When compared to the Malaysia Standard (2017), the current study revealed the pH values ranged between 4.75 -5.03 (Table 1). The mean pH values from the *T. laeviceps* presented the lowest pH value while the *G. thoracica* species obtained the highest pH value ( $P \leq 0.05$ ). These findings mirror those of the previous review by and Nordin et al. (2018) and Omar et al. (2019), who found that the pH of the stingless bee honey varies between 3.15 to 6.64. This is supported by Maringgal et al. (2019) who revealed similar acidic value for stingless bee honey from Malaysia with the pH values ranged from 4.25 to

5.5. Moreover, De Sousa *et al.* (2016) explained that the acidic value was corresponding to the balance of organic acid present in the honey, where the pH value varies and depends on the floral origin and the bee species. In addition, Ramón-Sierra *et al.* (2015) described that the pH value of honey is also affected by the fermentation of sugars to alcohol by microorganisms and being subjected to subsequent oxidation to carboxylic acids during storage. Suntiparapop *et al.* (2012) concluded that low pH value in *T. laeviceps* honey could contribute to their antimicrobial activity.

Bertoncelj *et al.* (2007) explained that colour is one of the physical observations that is related to the botanical origin, minerals, phenolic content, storage time, and temperature of the honey. The present study shows the value of colour intensity of honey produced by four different species of stingless bees ranged from 0.09-0.23 mAU (Table 1). The colour intensity values from *T. laeviceps* and *H. itama* had the highest values followed by the *L. terminata* and *G. thoracica* ( $P \leq 0.05$ ). Nevertheless, the ANOVA results showed that the colour intensity values are not statistically significant between the colour of honey produced by *T. laeviceps* and *H. itama*. However, there is a statistically significant difference for both species between the *L. terminata* and *G. thoracica*.

Similarly, Kek *et al.* (2014) also found the variations in colour intensity values of honey in different of bee species. Guerrini *et al.* (2009) described that the colour intensity of honey differs based on mineral content and pH. Meanwhile, Baretta *et al.* (2005) explained the variation of colour intensity of honey differs based on the geographical and the bee species. However, the colour intensity of honey also affected by a few factors like exposure to light, enzymatic reactions, and heat and storage time (De Sousa *et al.*, 2016).

### 3.4 Mineral elements

Finola *et al.* (2007) described that honey has various mineral composition. In addition, the authors added that mineral elements in honey are directly related to the geographical, environmental, and botanical aspects of the area where the honey is being produced. Figure 1 illustrated the mean value of different mineral elements content in honey produced by four different stingless bee species. It can be observed that Magnesium (Mg) is the most outstanding element in all analyses of honey, in which *G. thoracica* produced the highest Mg value at 83.02 mg/kg. A seminal study by Biluca *et al.* (2016) also found that the element of Mg in the stingless bee honey from Brazil is within the range 0.410-17.3 mg/100 g. This finding could be due to the different geographical location of the stingless bee to produce the honey. In a similar finding, Kek *et al.* (2017) claimed that the mean amount of Mg in honey that produced by *H. itama* from Malaysia was 33.81mg/kg.

Sodium (Na) is the second-largest mineral element that can be found in honey produced by four different stingless bee species ranging from 22.69-32.93 mg/kg. Honey produced by *T. laeviceps* has relatively high Na (32.93 mg/kg) followed by *L. terminata* (26.61 mg/kg), *G. thoracica* (24.78 mg/kg), and lastly *H. itama* (22.69 mg/kg). Pohl (2009) explained that the variations of Na content in honey might be because of flying area, nectar collection, and plant selection from the bees to produce the honey. These are in line with the finding of Fuenmayor *et al.* (2013), which described that mineral elements in honey are contingent upon the bee species apart from the botanical and the geographical origin. In addition, the authors also found the differences of mineral elements from different stingless bee species in Colombia.

Calcium (Ca) is the third abundant element and the

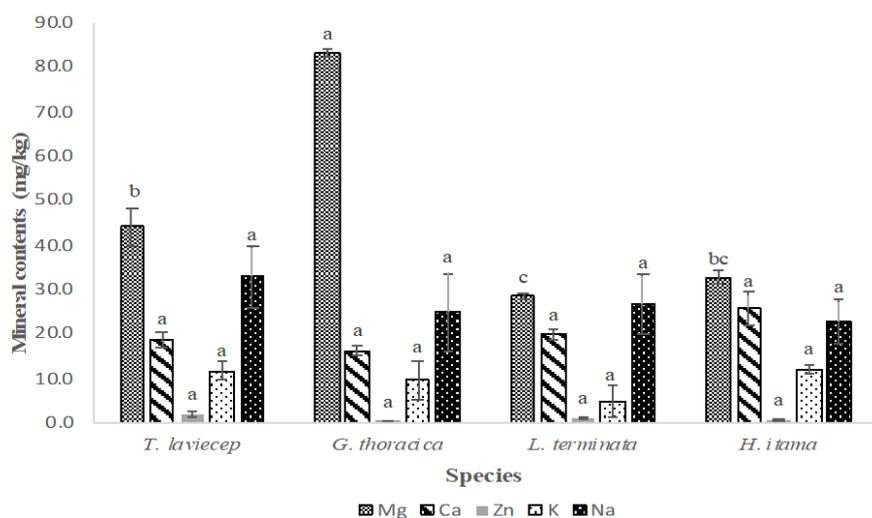


Figure 1. Mineral elements in four different species of stingless bee honey. Numbers are the mean±standard errors of four replications. Bars with the same letters above are not significantly different at  $P \leq 0.05$  by using Tukey test.

overall total average of Ca elements in four stingless bee honey species was 80.49 mg/kg. Honey that produced by *H. itama* possessed the highest in Ca (25.74 mg/kg) followed by *L. terminata* (19.83 mg/kg), *T. laeviceps* (18.73 mg/kg), and lastly *G. thoracica* (16.19 mg/kg). Meanwhile, the element of Potassium (K) obtained the fourth abundant mineral in all honey samples. The study indicated that honey being produced by *H. itama* had the highest in K (12.08 mg/kg) followed by *T. laeviceps* (11.64 mg/kg), *G. thoracica* (9.67 mg/kg), and lastly *L. terminata* (4.78 mg/kg).

Zinc (Zn) is detected as a minor element that is fewer than 4 mg/kg that was found in all honey samples. It can be noted that honey from the *T. laeviceps* revealed the highest in Zn value (2.07 mg/kg) followed by *L. terminata* (0.89 mg/kg), *H. itama* (0.65 mg/kg), and lastly *G. thoracica* (0.38 mg/kg). The ANOVA analysis also indicated that the element of Na, Ca, K, and Zn have no statistically significant difference between all the species of stingless bee. However, the element of Mg possessed statistically significant difference with all stingless bee species except for *H. itama*.

#### 4. Conclusion

The recent study showed that the TPC and TFC, colour intensity, antioxidant activity, pH, and the mineral elements of stingless bee honey can be elucidated by the natural disparities of the stingless bee species and the geographical location. This study has shown that different species of stingless bee resulted in different variation in phytochemical properties and mineral elements of honey. The evidence from this study also suggested that the differences in species were not sufficient enough to represent the statistically significant difference in phytochemical content and mineral elements of stingless bee honey. Different geographical location should be considered to obtain a more accurate finding on the phytochemical and mineral elements of the stingless bee honey. Future investigation and experiment into phytochemical, mineral elements and heavy metal of different species of stingless bee honey at different geographical regions in Malaysia is strongly recommended. This result can deliver a good database for establishing and identifying the international standard of stingless bee honey. The outcomes may also yield crucial data pertaining to stingless bee honey which could be referred to for future studies.

#### Conflict of interest

The authors have no competing interests to disclose.

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