

Research Article

Botanical Origin and Nutritional Values of Bee Bread of Stingless Bee (*Heterotrigona itama*) from Malaysia

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Bee bread is the bee-collected pollen with the mixture of honey and bee salivary enzymes, stored inside the bee hive. Malaysia has limited information on bee bread collected by the stingless bee *Heterotrigona itama*. Therefore, this study aims to determine the botanical origin and nutritional values of bee bread from *H. itama*. Melissopalynological analysis was performed using scanning electron microscopy (SEM) to determine the bee bread's botanical origin. The proximate analysis, water activity, sugar profile, amino acid profile, vitamin C content, mineral content, and heavy metal content of the bee bread were analyzed. From the results obtained, *Bidens pilosa* was found in bee bread from all sampling locations. Bee bread contained high protein (21.70–23.33%) and carbohydrate (57.06–58.89%) contents. Glucose was the predominant sugar found (average 11.499 g/100 g). Eight essential amino acids were quantified, and arginine was detected the highest. The major mineral element in the bee bread was potassium (average 6705.9 mg/kg), followed by phosphorus and magnesium. Toxic metals such as lead, mercury, cadmium, and arsenic were detected but within the safe limits permitted in food. The data obtained contribute towards expanding the knowledge on the nutritional information specifically for *H. itama* bee bread in Malaysia.

1. Introduction

Bee bread refers to the pollen collected by bee, added with nectar and bee salivary enzymes and stored inside a bee hive where it undergoes lactic acid fermentation. Information on bee bread as one of the bee products is overlooked with more highly acclaimed honey [1]. Acquisition of bee bread can be troublesome for some bee farmers because it requires the destruction of the bee hive for harvesting [2]. Thus, most studies were conducted on bee pollen of the honeybee *Apis mellifera* whereby bee pollen was obtained through the pollen trap installed at the bee hive's entrance. However, in the case of the stingless bee, bee bread is stored inside a cerumen pot. The bees will be able to repair the damage and rebuild the pots. Bee bread is a part of bee diet, mainly as a source of protein for bee larvae and young bee's development. Because of its nutritive properties, it can be considered a valuable food supplement for human consumption [2].

Bee bread is high in carbohydrates (24–34%), proteins (14–37%), and lipids (6–13%) [3] and contains other nutrients such as minerals [4–6] and vitamins [7]. Bee bread also provides the required essential amino acids which cannot be synthesized by humans [5, 8, 9]. However, the chemical composition of bee bread varies depending on the botanical origin, geographical location, climatic condition, soil type, beekeepers' activities, or storage treatments in commercial production [10, 11].

Phenolic compounds such as kaempferol and quercetin are commonly found in bee bread [2, 12, 13], and these compounds strongly contribute towards the bee bread's antioxidant properties [14]. Information on the bee bread's therapeutic properties is limited in the *in vitro* studies, whereby the antioxidant [7, 15–17], antibacterial [15, 18, 19], antitumour [12, 20], and ameliorating properties [21] were investigated, resulting in promising outcomes as functional food.

Limited studies have reported on the bee bread's nutritional values, especially from the stingless bee *Heterotrigona itama* in Malaysia. *H. itama* is the dominant among 35 stingless bee (locally known as "kelulut") species identified in Malaysia [22, 23]. Venturing into bee farming industry helps to generate side incomes, mainly gaining profit from honey production, but bee bread is underutilized by some bee farmers. Local price for bee bread can reach up to 95 USD/kg. Although Malaysia stingless beekeeping industry is progressively expanding, information on the Malaysian stingless bee's byproducts is still scarce [1], and Malaysian standards specifically for bee products are yet to be established. Currently, Wan Ismail et al. [24] only have reported the physicochemical analysis of *H. itama* bee bread from one sampling location in Eastern Malaysia.

Therefore, this study aims to identify the botanical origin of bee bread collected by *H. itama* and also its nutritional values (proximate analysis, sugar profile, amino acid profile, vitamin C content, mineral content, and heavy metal content). Sampling took place at four different geographical locations in West Malaysia.

2. Materials and Methods

2.1. Sample Collection. Bee bread from the stingless bee *H. itama* was harvested from commercial local bee farms at different geographical locations: MAEPS (2°58'56.8"N, 101°41'40.9"E), Ladang 10 UPM (2°59'31.2"N, 101°42'57.6"E), Batang Benar (2°58'54.7"N, 101°41'38.9"E), and Rinching Hilir (2°54'30.8"N, 101°49'06.0"E Johor), from April to May 2019. Fresh bee bread was collected from inside of the bee pots using spatula and stored in the universal container. The bee bread samples were then freeze-dried for 3 days before storing at 4°C for further analysis.

2.2. Melissopalynology. The bee bread was observed under scanning electron microscopy (SEM) according to the method of Ibrahim et al. [25]. The samples were mounted on the smooth surface with the adhesive tape on top of the aluminum stub. The samples were coated with gold (Baltec SDC 005 Sputter Coater) and then analyzed under SEM (JSM-IT100 InTouchScope). The images were captured with the voltage acceleration of 10 kV and flow of 1750 mA and observed at 1000–3500x magnifications. The pollen was identified using references from scientific articles and the online database at www.paldata.org.

2.3. Physicochemical Analysis. The proximate analysis was determined using the protocol of the Association of Official Analytical Chemists [26]. The protein content was determined using the Kjeldahl method and calculated using the conversion factor of 6.25 ($N \times 6.25$) (AOAC 981.10). The total lipid content was determined using the Soxhlet method (AOAC 991.36). The ash content was calculated according to the AOAC 923.03 method. The moisture content was determined by drying the samples in an air-dried oven (Mettler) at 100°C until a constant weight was achieved (AOAC 950.46). The total carbohydrate contents were

obtained by difference as follows: g carbohydrates = 100 g – (g moisture + g lipid + g protein + g ash).

Total energy was calculated using the following calculation: total energy (kcal/100 g) = (% protein \times 4) + (% carbohydrates \times 4) + (% fat \times 9).

For water activity, A_w , fresh wet bee bread was used. A_w was measured using a water activity meter.

2.4. Sugar Profile. The determination of sugar (glucose, fructose, maltose, and sucrose) was performed using HPLC coupled with an ELSD, according to the official method AOAC 982.14.

2.5. Determination of Vitamin C. Vitamin C was determined using the titration method with iodine according to the official method AOAC 967.21 (1997). 10 g of bee bread samples was homogenized in 100 mL metaphosphoric acid-acetic acid solution. The sample solution was directly titrated with 0.1 N iodine.

2.6. Amino Acid Profiling

2.6.1. Sample Preparation. Dried bee bread samples (0.1–0.2 g) were hydrolyzed in 5 mL of 6 N HCl at 110°C for 24 h. The hydrolysate was then added to 4 mL of 2.5 Mm alpha-aminobutyric acid (AABA) with 100 mL of water. The mixture was filtered with a filter paper. An aliquot was filtered through a syringe with the cellulose filter membrane (0.452 μ m).

2.6.2. Preparation of AccQ-Fluor Reagent for Derivatization. The AccQ-Fluor reagent was prepared according to the manufacturer's protocol. One milliliter of acetonitrile was added to a bottle containing AccQ-Fluor reagent powder (Waters). The mixture was vortexed before heating at 55°C for less than 10 min until it completely dissolved. The reconstituted AccQ-Fluor reagent was stored in a desiccator at room temperature for a maximum of one week.

2.6.3. Preparation of Amino Acid Standard. Forty microliters of 2.5 μ m/ml AABA (internal standard) were mixed with 40 μ L of amino acid standard "H" (Thermo Fisher) and 40 μ L of 2.5 μ m/mL hydroxyproline. Water (880 μ L) was added to the mixture. The standard solutions were stored at –20°C for up to 1 month.

2.6.4. Sample and Amino Acid Standard Derivatization. Prepared samples (10 μ L) were taken for derivatization by adding it with 100 μ L AccQ-Fluor reagent. For amino acid standard derivatization, 10 μ L of the prepared standard was added with 70 μ L borate buffer and 20 μ L AccQ-Fluor reagent. The mixture was vortexed and heated at 55°C for 10 min. The derivatives were stored at room temperature until 1 week.

Chromatographic separation was carried out in an AccQ-Taq column (3.9 mm \times 150 mm) fitted with a pre-column safeguard containing the same packing material

(12.5 mm × 4.6 mm, 4 μm). The column was thermostatted at 36°C, the flow rate was 1 mL/min, and the injection volume was 5 μL. The mobile phase contains AccQ-Tag Eluent A Concentrate and AccQ-Tag B or 60% acetonitrile. In terms of retention time, the composition of each peak was confirmed and determined in accordance with the external standard method. Before the gradient was started, the column was equilibrated in 100% A for 10 min. Fluorescence detection was carried out by λ irradiation at 250 nm excitation and 395 nm emission wavelength.

2.7. Mineral Content Analysis. Dried bee bread samples (0.5 g) were digested with 3 mL of 60% nitric acid and 2 mL of 30% hydrogen peroxide inside a tetrafluormethaxil (TFM) vessel of the microwave digester (Milestone) for 18 min. Then, the vessel was cooled down for 10 min. The solution was collected from inside of the vessel and poured into a 50 mL volumetric flask with 1 mL nitric acid and diluted with deionized water to 50 mL.

The mineral composition of bee bread was then analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (ELAN 9000, PerkinElmer) after microwave-assisted acid digestion. The concentrations of mineral elements (Ca, Fe, K, Mg, Mn, Na, P, and Se) were determined in bee bread samples. The limit of detection was 0.001 mg/kg.

2.8. Heavy Metal Analysis. Dried bee bread samples (0.5 g) were digested with 3 mL of 60% nitric acid and 2 mL of 30% hydrogen peroxide inside a tetrafluormethaxil (TFM) vessel of the microwave digester (Milestone) for 18 min. Then, the vessel was cooled down for 10 min. The solution was collected from inside of the vessel and poured into a 50 mL volumetric flask with 1 mL nitric acid and diluted with deionized water up to 50 mL.

The heavy metal in bee bread was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (ELAN 9000, PerkinElmer) after microwave-assisted acid digestion. The concentrations of heavy metals (As, Hg, Sb, Cd, Pb, Sn, Cu, Zn, and Cr) were determined. The limit of detection was 0.001 mg/kg.

2.9. Statistical Analysis. The samples were analyzed in triplicate analysis. Statistical analysis was performed using Minitab version 17. One-way ANOVA was used to compare mean in each analysis. If the ANOVA test indicated the significant result ($P < 0.05$), then the significant means were separated using Tukey's test.

3. Results and Discussion

3.1. Melissopalynology. Melissopalynological analysis provides the information on the plant visited by the bees. Table 1 shows the pollen analysis of bee bread from different geographical locations. The melissopalynological analysis of stingless bee (*H. itama*) bread taken from four different geographical locations showed that a total of 10 pollen species from 5 families were identified. The families were

Thymelaeaceae, Rutaceae, Arecaceae, Asteraceae, and Fabaceae, with the last two being predominant. *Bidens pilosa* was present in all bee bread samples. The identified pollen grains are shown in Figure 1.

Bidens pilosa is classified as a perennial herb, found across the tropical region. *B. pilosa* has attracted a wide range of insects from bees to wasps, bee flies, and butterflies [27]. It is one of the dominant plant pollen collected by *H. itama* in Terengganu, Malaysia [28]. Thakodee et al. [29] also reported *B. pilosa* as a plant visited by the stingless bee *T. pagdeni* in northern Thailand.

B. pilosa flower is small and white in colour, which is one of the factors attracting the pollinator. *H. itama* in this study largely foraged pollen from white and yellow colour flowers. This is in accordance with research by Kiew and Muid [30] whereby 66% of bee pollen in Malaysia originated from a plant with white and yellow flowers. Similarly, Ghazi et al. [31] found white and cream flowers attract *H. itama* the most at Tasik Kenyir, Terengganu, Malaysia. Apart from colour, size also plays an important role. Small flowers such as *Mimosa pudica* were favoured by *H. itama* [32].

3.2. Physicochemical Analysis. Table 2 displays the proximate analysis, energy value, and water activity of bee bread of the stingless bee *H. itama*. Carbohydrate was the most available macronutrient (58.16 ± 0.69 g/100 g) in *H. itama* bee bread. The carbohydrate level was above the minimum level required by bee pollen, 40 g/100 g, set by Campos et al. [33]. It was similar to that of bee bread of the stingless bee *T. laeviceps* (58.7 g/100 g) but higher than that of bee bread of *L. terminata* (53.4 g/100 g), *L. flavibasis* (53.3 g/100 g), and *T. testaceitarsis* (43.1 g/100 g) from Thailand [34], *H. itama* (55.1%) from Perak, Malaysia [24], and *M. seminigra* (25.66%), *M. interrupta* (44.27%), and *T. angustula* (45.98% and 46.68%) from Brazil [13, 35]. In Philippines, the carbohydrate content of *Tetragonula biroi* bee bread was in the range of 53.05 to 59.94 g/100 g [4].

Bee bread is mostly composed of carbohydrates such as glucose, fructose, sucrose, starch, and also pectin, which build the pollen wall [33, 36]. In the process of making bee bread, the carbohydrate content in bee bread is increased due to the addition of nectar and honey with the fresh pollen. This was observed by Human and Nicolson [9] in stored bee pollen of *Aloe greatheadii* as compared to fresh pollen.

The protein values of the bee bread samples in the present study were between 21.70 and 23.33 g/100 g, above 15 g/100 g, which is the protein standard level in bee pollen [33]. This result is in agreement with the protein values of bee bread from other stingless bee species such as *T. angustula* (22.97% and 22.43%), *M. interrupta* (24.00%), and *T. biroi* Friese (21.69 g/100 g) [4, 13, 35]. However, Wan Ismail et al. [24] found higher protein levels in *H. itama* bee bread (47.4%). In contrast, Chuttong et al. [34] reported lower protein contents of 15.5 g/100 g (*T. laeviceps*), 17.9 g/100 g (*T. testaceitarsis*), 14.3 g/100 g (*L. terminata*), and 16.7 g/100 g (*L. flavibasis*).

Other bee species such as honeybee also reported variation in the protein range whereby bee bread of Colombian

TABLE 1: Melissopalynological analysis of pollen from *H. itama* bee bread based on different geographical locations.

Geographical locations	Scientific name	Family	Common name
Ladang 10 UPM	<i>Mimosa pudica</i>	Fabaceae	Pokok semalu
	<i>Sphagneticola trilobata</i>	Asteraceae	—
	<i>Bidens pilosa</i>	Asteraceae	Pokok biden
MAEPS	<i>Bidens pilosa</i>	Asteraceae	Pokok biden
	<i>Cassia</i> sp.	Fabaceae	—
	<i>Areca catechu</i>	Arecaceae	Pokok pinang
	<i>Peltophorum pterocarpum</i>	Fabaceae	Jemberlang laut
	<i>Phaleria capitata</i>	Thymelaeaceae	—
Rinching Hilir	<i>Bidens pilosa</i>	Asteraceae	Pokok biden
	<i>Cassia siamea</i>	Fabaceae	Johar
Batang Benar	<i>Bidens pilosa</i>	Asteraceae	Pokok biden
	<i>Citrus aurantifolia</i>	Rutaceae	Limau nipis
	<i>Ageratum conyzoides</i>	Asteraceae	Pokok tahi ayam

Apis mellifera had 23.1% protein [37], slightly higher than that of Indian *Apis dorsata* (16.86–17.45 g/100 g) and Indian *Apis mellifera* (17.19–19.10 g/100 g) [38]. The protein content in bee bread varied according to the plant source [39, 40] but can be unpredictable because of the addition of sugar by bee [41].

The lipid content of *H. itama* bee bread was reported between 4.64 and 5.95 g/100 g. Based on Campos et al.'s [33] standard, lipid should not be less than 1.5 g/100 g. This result is in agreement with the findings on bee bread of other stingless bee species such as *T. biroi* Friese (2.64–5.43 g/100 g) from Philippines [4] and *T. testaceitarsis* (5.4 g/100 g), *L. terminata* (5.3 g/100 g), and *L. flavibasis* (4.9 g/100 g) from Thailand [34] but lower than that of *T. laeviceps* (7.4 g/100 g) [34] and *M. seminigra* (10.81%) and *M. interrupta* (6.47%) from Brazil [35].

Lipid comprised the pollen outermost wall known as the pollenkit [42, 43]. It is present in the pollen grains with high variability, which can be explained due to the various plant species visited by the bees as demonstrated by Margaoan et al. [44]. Lipid composition in the fresh pollen can be altered after collected by bees [9], possibly from the addition of the bee glandular enzyme to process bee pollen to bee bread.

Ash was the least macronutrient available in *H. itama* bee bread with the average value of 2.54 ± 0.12 g/100 g. According to Campos et al. [33], the ash content should not be more than 6 g/100 g. This result corroborates with other studies which found the ash content to be in the similar range in bee bread of the stingless bee *T. biroi* from Philippines (3.58 g/100 g) [4], *H. itama* from Malaysia (1.70%) [24], and *T. laeviceps* (2.3 g/100 g), *T. testaceitarsis* (2.2 g/100 g), *L. terminata* (1.8 g/100 g), and *L. flavibasis* (2.2 g/100 g) from Thailand [34].

The energy values of *H. itama* bee bread obtained were between 367.33 and 371.00 kcal/100 g. These values were higher compared to those of bee bread samples of *M. seminigra* (350.57 kcal/100 g) and *M. interrupta* (331.33 kcal/100 g) from Brazil [35] and *T. biroi* (216 to 266 kcal/100 g) from Philippines [4] but lower than those of bee bread from Morocco (396.2 kcal/100 g) [21].

Dried *H. itama* bee bread in this study had moisture between 11.09 and 12.51%, above the standard limit of 6–8 g/

100 g [33]. It is comparable to dried *T. biroi* bee bread with the moisture content of 14.15 to 16.73% [4], but it has lower moisture content than 31.7 g/100 g reported previously from *T. testaceitarsis* in Thailand by Chuttong et al. [34]. Compared to dried bee bread, fresh bee bread has higher moisture content. Pollen has hygroscopic properties, making it absorb water readily from the surrounding. Fresh bee bread can have moisture content up to 53.39% based on studies by Rebelo et al. [35] on fresh pot pollen of stingless bees *M. seminigra* and *M. interrupta* in Brazil. Therefore, bee bread required adequate drying parameters to achieve the desired moisture content to prevent spoilage during storage.

However, moisture content cannot determine the food stability alone. Food with a similar moisture content has different perishability because water molecules encounter different ways to interact with other food constituents [45]. Thus, water activity (A_w) is the better indicator. High A_w favours the microbial growth on food. In this study, A_w of fresh bee bread of *H. itama* was recorded in the range of 0.729 to 0.852. In Brazil, A_w of fresh *M. seminigra* and *M. interrupta* bee bread was reported to be high, with 0.910 and 0.850, respectively [35]. Alves et al. [46] also reported high A_w in bee bread of *M. scutellaris* from Brazil with the values of 0.920 (collected in the dry season) and 0.930 (collected in the rainy season). However, dehydrated bee bread has lower A_w as can be seen in dried *T. biroi* bee bread (0.600 to 0.690) [4].

Microbial growth is believed to occur at A_w above 0.600 [47]. The preservation method such as drying until A_w goes below this level can help prolong the shelf-life of bee bread. Because fresh bee bread has higher A_w , this increases the chances for growth of either beneficial bacteria, pathogenic bacteria, fungi, or mold [48]. Eventually, this contributes to the presence of mycotoxins such as aflatoxin and ochratoxin, which has been reported in various pollen [49]. Therefore, this drying parameter should be critically observed to prevent mycotoxin accumulation during storage at low temperature, to avoid the moisture increase throughout shelf-life. Aside from water activity, other factors such as bee bread's high acidity can reduce the risk of pathogenic contamination in bee bread.

The minimum daily recommended intake of bee bread for adults is 20 g [3]. Therefore, based on this study, a serving

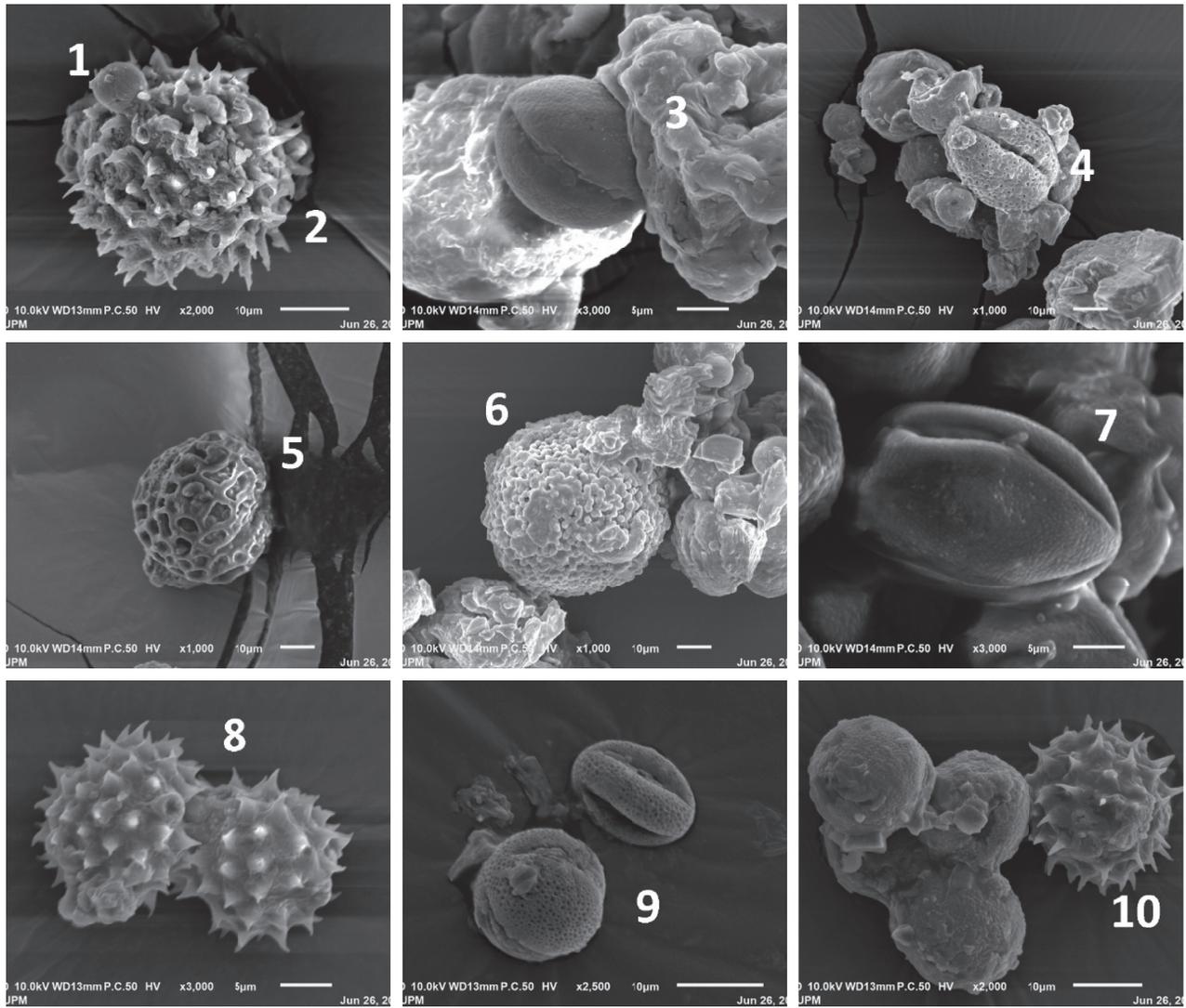


FIGURE 1: Pollen grains from *H. itama* bee bread: (1) *Mimosa pudica*, (2) *Sphagneticola trilobata*, (3) *Cassia* sp., (4) *Areca catechu*, (5) *Peltophorum pterocarpum*, (6) *Phaleria capitata*, (7) *Cassia siamea*, (8) *Ageratum conyzoides*, (9) *Citrus aurantifolia*, and (10) *Bidens pilosa*.

TABLE 2: Physicochemical analysis of bee bread samples from four different geographical regions.

Sample	Protein (%) ¹	Total lipid (%) ¹	Total carbohydrate (%) ¹	Ash (%) ¹	Moisture (%) ¹	Energy (kcal/100 g) ¹	Water activity, A_w
Ladang 10 UPM	22.03 ± 2.23 ^a	5.95 ± 0.10 ^a	57.06 ± 2.09 ^a	2.46 ± 0.02 ^b	12.51 ± 0.07 ^a	369.67 ± 0.58 ^{ab}	0.840 ± 0.033 ^a
Batang Benar	23.33 ± 0.48 ^a	4.64 ± 0.04 ^c	58.13 ± 0.43 ^a	2.47 ± 0.05 ^b	11.42 ± 0.29 ^{bc}	367.33 ± 1.53 ^b	0.729 ± 0.004 ^a
MAEPS	22.01 ± 0.08 ^a	5.27 ± 0.18 ^b	58.89 ± 0.28 ^a	2.74 ± 0.01 ^a	11.09 ± 0.07 ^c	371.00 ± 1.00 ^a	0.852 ± 0.015 ^b
Rinching Hilir	21.70 ± 0.08 ^a	5.48 ± 0.16 ^b	58.56 ± 0.12 ^a	2.48 ± 0.01 ^b	11.78 ± 0.25 ^b	370.67 ± 1.53 ^a	0.835 ± 0.010 ^a
Average	22.26 ± 0.63	5.34 ± 0.47	58.16 ± 0.69	2.54 ± 0.12	11.70 ± 0.53	369.67 ± 1.44	0.814 ± 0.049

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with different letters in the same column are statistically different ($P < 0.05$). ¹Dry weight.

of *H. itama* bee bread can contain approximately 11.63 g carbohydrates, 4.45 g proteins, 1.07 g lipids, 0.51 g minerals, and 73.93 kcal.

3.3. Sugar Profile. Table 3 demonstrates the sugar content of glucose, fructose, maltose, and sucrose in bee bread of the stingless bee *H. itama*. Glucose was the most abundant sugar

TABLE 3: Sugar content (fructose, glucose, sucrose, and maltose) in bee bread samples.

Sampling	Fructose (g/100 g)	Glucose (g/100 g)	Sucrose (g/100 g)	Maltose (g/100 g)
Ladang 10 UPM	1.488 ± 0.140 ^a	10.270 ± 0.140 ^a	1.984 ± 0.001 ^a	0.794 ± 0.001 ^c
MAEPS	0.795 ± 0.000 ^b	11.830 ± 1.550 ^a	1.293 ± 0.141 ^b	1.491 ± 0.140 ^b
Rinching Hilir	0.396 ± 0.000 ^b	11.700 ± 1.960 ^a	0.595 ± 0.000 ^c	0.694 ± 0.140 ^c
Batang Benar	1.296 ± 0.141 ^a	12.397 ± 0.980 ^a	2.094 ± 0.141 ^a	1.994 ± 0.000 ^a
Average	0.994 ± 0.428	11.549 ± 0.784	1.492 ± 0.602	1.244 ± 0.531

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with different letters in the same column are significantly different ($P < 0.05$).

found in *H. itama* bee bread, which ranged from 10.270 to 12.397 g/100 g. It is higher than glucose in the pot pollen of *T. biroi* from Philippines which ranged from 0.250 to 1.940 g/100 g [4]. However, the same authors discovered higher contents of fructose and sucrose of 7.31 g/100 g and 12.77 g/100 g, respectively. In this reported study, the fructose level was within 0.795 to 1.488 g/100 g, while the sucrose level was between 1.293 and 2.094 g/100 g. High glucose content over fructose was also obtained by Bertoneclicj et al. [50] in Slovenian bee pollen collected by *A. mellifera carnica*.

Similarly, Bobiş et al. [51] found fructose (18.95 g/100 g) and glucose (11.54 g/100 g) in high concentration, but no sucrose was detected in bee bread of honeybee. In bee bread samples from Romania and India, the fructose and glucose concentrations were very high; again, no sucrose was detected [52]. Based on previous studies, the highest sugar in bee bread of the stingless bee was mannitol. Silva et al. [5] quantified mannitol as the highest sugar found in *Melipona subnitida* bee bread collected in Brazil with the value of 31 g/100 g. Meanwhile, Wan Omar et al. [53] also identified mannitol as the main sugar in bee bread of *Trigona thoracica* (54.34%), *Trigona apicalis* (39.11%), and *Trigona itama* (33.05%) in Malaysia.

The sugar content varied between bee bread samples upon origin, regions, and time harvesting [50]. Bee bread constitutes bee salivary glands which contain different enzymes, depending on the bee species. Stingless bees *Meliponini*, *Trigonini*, and *Scaptotrigona* spp. have been shown to secrete α - and β -amylase and α -glucosidase [54], resulting in breakdown of polysaccharides into simple sugar. Sugar, such as glucose and fructose, is required by bacteria to ferment bee pollen into bee bread. It is digested into ethanol, lactic acid, and carbon dioxide by lactic acid bacteria. Silva et al. [5] also suspected glucose and fructose to be converted into mannitol after mixing with bee salivary enzymes. In turn, this possibly reduces the sugar content available in bee bread into varying degrees.

3.4. Amino Acid Profile. Table 4 shows the amino acid content in *H. itama* bee bread. The amino acid was quantified based upon the chromatogram profile (see Supplementary Materials (available here)). From Table 4, it can be seen that *H. itama* bee bread contained 8 essential amino acids, 4 conditionally essential amino acids, and 5 nonessential amino acids. Arginine was the predominant amino acid, with the value of 2.334 g/100 g, followed by phenylalanine, 2.288 g/100 g. Other studies also showed different

concentrations of amino acid composition. Chutong et al. [34] reported lysine as the most abundant amino acid (2.46 g/100 g) in *Tetragonula laeviceps* pot pollen. In addition, the concentration of most of the amino acids in the *T. laeviceps* pot pollen generally lowered than that in this study.

In another study by Human and Nicolson [9], the authors identified glutamic acid (10.78 g/100 g) as the highest amino acid content in monofloral stored pollen of *Aloe greatheadii*. Silva et al. [5] found high concentration of proline in bee bread of Brazilian *M. subnitida*. The difference in amino acid composition of bee bread is highly attributed to the botanical origin of the collected pollen which could also be affected by external factors such as seasons and geographical locations [34].

In general, amino acids are present in flower pollen and vary in levels depending on the plant visited by the bees. However, amino acid in fresh pollen is observed to be increased after being foraged by bees. Degrandi-Hoffman et al. [55] observed higher contents of most amino acids in European honeybee bread as opposed to bee pollen. Although Human and Nicolson [9] recorded increased amino acid contents from fresh pollen, bee pollen, to stored pollen, these differences were insignificant ($P = 1.00$).

Increased amino acid content in bee pollen was suggested by Kieliszek et al. [3] as a result of degradation of pollen protein, which led to formation of more peptides and amino acids. Bee bread is a niche to various bacteria such as *Bacillus* and lactic acid bacteria (LAB), but pollen proteolysis by indigenous bacteria is still debatable. Janashia et al. [56] detected no proteolytic activity of LAB isolated from honeybee bread using pollen as the substrate as compared to using milk. In different studies by Ngalimat et al. [57], the authors detected milk proteolytic activity by *Bacillus* spp. from *H. itama* nest products, but pollen proteolysis using *Bacillus* was not investigated.

However, just as amino acid is synthesized and accumulated in pollen, it could also be reduced through microbial activity whereby amino acids were utilized as carbon and energy sources [55]. Not only are amino acids significant as energy sources, but also the catabolism of amino acids is an important role of LAB in adapting to stress [58].

3.5. Mineral Content. The mineral contents of bee bread are shown in Table 5. The most abundant mineral in bee bread was potassium (average 6524.9 mg/kg), followed by phosphorus (6402.3 mg/kg) and magnesium (1635.4 mg/kg). Selenium and manganese were the least minerals with the

TABLE 4: Amino acids in bee bread of the stingless bee *H. itama*.

Amino acid (g/100 g)	Geographical locations				
	Ladang 10 UPM	Batang Benar	MAEPS	Rinching Hilir	Average
<i>Essential amino acids</i>					
Phenylalanine	2.796 ± 0.567 ^a	2.311 ± 0.190 ^{ab}	1.843 ± 0.084 ^b	2.202 ± 0.232 ^{ab}	2.288 ± 0.341
Valine	1.117 ± 0.051 ^a	1.101 ± 0.221 ^a	1.004 ± 0.019 ^b	1.109 ± 0.028 ^a	1.083 ± 0.046
Histidine	1.151 ± 0.168 ^a	1.006 ± 0.159 ^{ab}	0.778 ± 0.070 ^b	1.044 ± 0.111 ^{ab}	0.995 ± 0.136
Methionine	0.525 ± 0.091 ^a	0.484 ± 0.065 ^a	0.365 ± 0.047 ^a	0.407 ± 0.068 ^a	0.445 ± 0.063
Isoleucine	0.760 ± 0.180 ^a	0.938 ± 0.052 ^a	0.881 ± 0.018 ^a	0.825 ± 0.141 ^a	0.851 ± 0.066
Leucine	1.834 ± 0.083 ^a	1.677 ± 0.096 ^{ab}	1.606 ± 0.043 ^b	1.642 ± 0.030 ^b	1.690 ± 0.087
Threonine	2.001 ± 0.282 ^a	1.763 ± 0.188 ^a	1.519 ± 0.050 ^a	1.721 ± 0.188 ^a	1.751 ± 0.171
Alanine	1.036 ± 0.110 ^a	1.012 ± 0.056 ^a	1.004 ± 0.352 ^a	1.0597 ± 0.090 ^a	1.028 ± 0.022
<i>Conditionally essential amino acids</i>					
Arginine	2.660 ± 0.296 ^a	2.300 ± 0.276 ^{ab}	1.959 ± 0.117 ^b	2.416 ± 0.107 ^{ab}	2.334 ± 0.252
Tyrosine	1.634 ± 0.098 ^a	1.484 ± 0.145 ^a	1.100 ± 0.068 ^b	1.370 ± 0.152 ^{ab}	1.397 ± 0.195
Glycine	1.414 ± 0.156 ^a	1.243 ± 0.069 ^a	1.479 ± 0.064 ^a	1.121 ± 0.046 ^b	1.314 ± 0.141
Proline	1.711 ± 0.106 ^a	1.573 ± 0.087 ^{ab}	1.478 ± 0.052 ^b	1.530 ± 0.051 ^{ab}	1.573 ± 0.086
<i>Nonessential amino acids</i>					
Hydroxyproline	0.570 ± 0.126 ^a	0.581 ± 0.086 ^a	0.259 ± 0.054 ^a	0.415 ± 0.191 ^a	0.456 ± 0.131
Serine	2.182 ± 0.426 ^a	2.152 ± 0.137 ^a	1.783 ± 0.058 ^a	2.055 ± 0.121 ^a	2.043 ± 0.157
Glutamic acid	1.638 ± 0.426 ^a	1.491 ± 0.071 ^a	1.658 ± 0.123 ^a	1.742 ± 0.013 ^a	1.632 ± 0.090
Aspartic acid	1.577 ± 0.352 ^a	1.400 ± 0.115 ^a	1.381 ± 0.009 ^a	1.363 ± 0.101 ^a	1.430 ± 0.857
Lysine	0.735 ± 0.109 ^a	0.617 ± 0.106 ^a	0.704 ± 0.049 ^a	0.752 ± 0.062 ^a	0.702 ± 0.052

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with different letters in the same row are significantly different ($P < 0.05$).

TABLE 5: Mineral content (mg/kg) of *H. itama* bee bread samples.

Mineral (mg/kg)	Geographical locations				
	Ladang 10 UPM	Batang Benar	Rinching Hilir	MAEPS	Average
Calcium	1508.30 ± 142.90 ^a	1646.45 ± 8.97 ^a	1455.20 ± 35.10 ^a	1579.28 ± 7.32 ^a	1547.31 ± 72.21
Iron	127.72 ± 5.54 ^{ab}	120.31 ± 6.37 ^b	119.52 ± 7.63 ^b	138.17 ± 6.63 ^a	126.43 ± 7.49
Potassium	6394.0 ± 158.5 ^b	6171.8 ± 127.4 ^{bc}	5981.9 ± 50.2 ^c	7551.9 ± 50.2 ^a	6524.9 ± 610.6
Magnesium	1561.9 ± 140.6 ^b	1441.8 ± 27.3 ^b	1537.7 ± 23.6 ^b	2000.2 ± 21.6 ^a	1635.4 ± 215.4
Manganese	71.665 ± 0.986 ^{ab}	57.490 ± 12.250 ^b	37.277 ± 0.699 ^c	80.195 ± 0.657 ^a	61.66 ± 16.24
Sodium	155.51 ± 8.15 ^a	115.70 ± 14.23 ^b	142.64 ± 1.29 ^a	144.93 ± 1.35 ^a	139.70 ± 14.68
Zinc	60.3000 ± 0.6870 ^{ab}	59.0700 ± 4.0700 ^b	44.9100 ± 12.1900 ^b	78.1900 ± 5.8200 ^a	60.6175 ± 11.8112
Phosphorus	6392.02 ± 10.37 ^b	6143.3 ± 102.6 ^c	6496.7 ± 60.5 ^{ab}	6577.1 ± 42.6 ^a	6402.28 ± 163.29
Selenium	0.1890 ± 0.0662 ^b	0.1860 ± 0.0484 ^b	0.4447 ± 0.0234 ^a	0.2333 ± 0.0362 ^b	0.2633 ± 0.1064

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with different letters in the same row are statistically different ($P < 0.05$).

values of 0.20 mg/kg and 69.79 mg/kg, respectively. Significance differences ($P < 0.05$) were observed in mineral contents among different geographical locations except for iron and selenium.

From the literature review, potassium has been reported as the predominant mineral in bee bread of stingless bees. In Thailand, pot pollen of *T. testaceitarsis*, *L. terminata*, *L. flavibasis*, and *T. laeviceps* contained high potassium, from 4594.7 mg/kg to 5656.0 mg/kg. Belina-Aldemita et al. [4] also found high amount of potassium and sodium in pot pollen of *T. biroii* in Philippines. This result is also in agreement with those of Bakour et al. [21] as potassium was the main mineral in Moroccan bee bread with the value of 338 mg/100 g, followed by phosphorus (251 mg/100 g).

Multiple other studies also provided similar findings to this study. Potassium was the highest mineral content in bee pollen of *M. subnitida* in Brazil [5]. This is also supported by

Kostic et al. [59] who discovered high abundance of potassium (average of 3391 mg/kg) followed by calcium (1425 mg/kg) and magnesium (749 mg/kg) in bee pollen from Serbia. In a study by Somerville and Nicol [60], 34 floral species of honeybee-collected pollen in Australia were analyzed, and it was discovered that potassium was the highest mineral available with the mean concentration of 5530 mg/kg followed by phosphorus (4600 mg/kg). Potassium was also abundant (3605.37–6033.69 mg/kg) in honeybee bread in Romania [6]. This study obtained higher amounts of potassium and phosphorus in *H. itama* bee bread than other studies previously mentioned.

Potassium is required for pollen germination and flowering [61] and is acquired through soil, air, or water. Because bee bread is moisturized and agglutinated using nectar, more minerals are added and accumulated in the flower pollen. Plant type was suggested to affect the mineral

content in pollen but not the soil type [59]. In animal cells, potassium involves in various physiological processes encompassing regulation of osmotic pressure, muscle contraction, cell membrane function, and Na^+/K^+ -ATPase. Meanwhile, phosphorus is an essential element to synthesize ATP, DNA, and phospholipid membrane of animal cells [62]. This provides necessities for the bee growth and also indigenous microbes in bee bread.

3.6. Vitamin C Content. Table 6 displays the vitamin C content in bee bread. Vitamin C ranged from 0.1087 to 0.1152 mg/g. No significance difference was observed in the vitamin C content between different geographical locations. Based on the daily recommended intake (DRI) of vitamin C, the bee bread sample provides low vitamin C content for adults.

There are limited data reported on the vitamin C content in bee bread. Instead, multiple studies have been performed on vitamin C analysis in the bee pollen. Studies showed a higher vitamin C content in bee pollen as indicated by Sattler et al. [63]. The authors recorded the average value of 0.2623 mg/g for vitamin C in 21 bee pollen samples from Southern Brazil, with two samples been implied as the dietary source for vitamin C. Meanwhile, Oliveira, Moriya, Azedo, and Almeida-Muradian [64] also quantified higher vitamin C in fresh bee-collected pollen of *Apis mellifera* from Sao Paolo, Brazil, in the range between 0.274 and 0.559 mg/g.

Because pollen contains high moisture content, the method of preservation and its parameter could affect vitamin C stability. For example, vitamin C was not detected in heat-dried bee pollen samples of *Apis mellifera* from Brazil [65]. In contrast, Melo et al. [64] obtained higher vitamin C in dehydrated *Apis mellifera* bee pollen as compared to fresh bee pollen. However, in this study, the bee bread was freeze-dried whereby low temperature was applied on the samples, reducing possible vitamin C denaturation through freeze-drying [66]. Because the drying parameters were not optimized in this study, the amount of vitamin C loss in the bee bread samples was unclear.

Other factors affecting vitamin C stability are storage age and storage condition, which result in loss of vitamin C as can be seen in the dehydrated bee pollen of *Apis mellifera* in Brazil [67]. Storing in a freezer for at least 6 months has reduced vitamin C at most 26%. Oliveira et al. [64] also suggested the botanical origin, soil type, or climate condition to influence the vitamin C content in pollen.

3.7. Heavy Metal. Table 7 depicts the heavy metal content in bee bread. Heavy metals such as mercury, arsenic, cadmium, and lead, which are toxic in trace amounts, were detected in all of the bee bread samples. The heavy metal levels in bee bread were statistically different ($P < 0.05$) between geographical locations.

Mercury (Hg) toxicity imposed risk to multiple human systems such as neurological, nephrological, immunological, cardiac, motor, and reproductive systems [68]. Hg levels in

the bee bread samples of the current study were below the Hg limit in pollen set in [33], which were less than 0.03 mg/kg, except for the UPM sample (0.0307 mg/kg). Low Hg levels were also observed in bee bread of Moldova and Moscow (< 0.00036 mg/kg). Similarly, the mean Hg concentration in pollen loads from the Poland airfield area reported by Roman [69] was 0.0066 mg/kg, but one pollen sample was detected with a high Hg level (0.0427 mg/kg).

Arsenic (As) is used in agricultural management as a pesticide, herbicide, algicide, and wood preservative. It is potentially carcinogenic, and its toxicity leads to organ dysfunction [70]. The accepted limit of As in bee pollen is 0.5 mg/kg [33]. The mean As concentration in bee bread collected in this study was obtained under this limit. Golubkina et al. [71] also reported a low As level in bee bread from Poland. However, in a study by Altunatmaz [72], high As concentration (1.037 mg/kg) was recorded in one of the Turkey bee pollen samples. A study by Roman [69] also found high As concentration in individual samples of bee pollen, measured at 1.517 mg/kg.

Cadmium (Cd) is used in industrial activities and present in trace amounts in certain food, but its toxicity can lead to severe pulmonary and gastrointestinal irritation [70]. The Cd limit in bee pollen is less than 0.1 mg/kg [33]. In this study, samples from Batang Benar, Rincing Hilir, and MAEPS recorded Cd concentration above the limit with values of 0.1073, 0.1497, and 0.4033 mg/kg, respectively. Still, these are in the acceptable range according to the Cd limit (< 1 mg/kg) for food based on the Malaysian Food Regulation, 1985 [73]. This is similar to the results of Golubkina et al. [71] where bee bread from the suburb area in Moscow had a mean Cd value of 0.110 mg/kg. However, this is lower than the Cd concentration found in bee pollen collected at the Poland military airfield in 2005 with the highest value of 1.798 mg/kg.

Based on Table 7, lead (Pb) in the bee bread samples was recorded between 0.1980 and 1.0127 mg/kg. Samples from MAEPS had exceeded the acceptable limit of Pb in bee pollen (0.5 mg/kg) set by Campos et al. [33] but have Pb content less than the Pb limit set by the Malaysian Food Regulation, 1985 (2 mg/kg). High Pb content was also reported in bee pollen from the industrial part of Jordan, with the value of 2.567 mg/kg [74]. However, Roman [69] detected a higher Pb content in a bee pollen sample from the Poland agricultural region with the value of 3.900 mg/kg. In another study by Golubkina et al. [71], bee bread from unpolluted and suburb areas in Russia contained a lower Pb level, which was 0.210 and 0.341 mg/kg, respectively.

Heavy metals are taken up by plants through various routes such as soil, air, and water. The increase of anthropogenic activities such as pesticide usage, land use, and industrial activities has led to the increase of heavy metal pollution in the environment. These activities are different based on the geographical landscapes such as urban, agricultural, and industrial areas, which eventually lead to different levels of heavy metal pollution in the pollen [69, 74]. However, pollen's ability to bioaccumulate heavy metals has created an opportunity for the pollen to become a bio-indicator to detect heavy metal pollution in the environment.

TABLE 6: Vitamin C content in *H. itama* bee bread.

Sample	Vitamin C (mg/g) ¹	% DRI (men) ²	% DRI (women) ²
Ladang 10 UPM	0.1087 ± 0.0110 ^a	2.41	2.90
Batang Benar	0.1051 ± 0.0057 ^a	2.34	2.80
Rinching Hilir	0.1142 ± 0.0082 ^a	2.54	3.05
MAEPS	0.1152 ± 0.0079 ^a	2.56	3.07
Average	0.1108 ± 0.0041		

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with the different letters are statistically different ($P < 0.05$). ¹Dry weight; ²90 mg/day for men and 75 mg/day for women. Values are based on bee pollen intake of 20 g/day as suggested by Kieliszek et al. [3].

TABLE 7: Heavy metal (mg/kg) composition in *H. itama* bee bread and comparison with the maximum acceptable level of metals.

Heavy metal (mg/kg)	Geographical locations					Bee pollen standard ¹	Food regulation, 1985 (mg/kg) ²
	UPM (residential area)	Batang Benar (residential area)	Rinching Hilir (residential area)	MAEPS (agricultural area)	Average		
Arsenic	0.0867 ± 0.0038 ^b	0.0177 ± 0.1002 ^c	0.0773 ± 0.0084 ^b	0.1570 ± 0.0157 ^a	0.0847 ± 0.0494	<0.5	1
Mercury	0.0307 ± 0.0025 ^a	0.0260 ± 0.0036 ^{ab}	0.0237 ± 0.0021 ^b	0.0213 ± 0.0058 ^b	0.0254 ± 0.0035	<0.03	0.05
Antimony	0.0187 ± 0.0006 ^b	0.0217 ± 0.0055 ^{ab}	0.0267 ± 0.0064 ^{ab}	0.0323 ± 0.0006 ^a	0.0249 ± 0.0052	NA	1
Cadmium	0.0653 ± 0.0021 ^d	0.1073 ± 0.0056 ^c	0.1497 ± 0.0124 ^b	0.4033 ± 0.0222 ^a	0.1810 ± 0.1312	<0.1	1
Lead	0.1980 ± 0.0096 ^d	0.2407 ± 0.0067 ^c	0.3307 ± 0.0101 ^b	1.0127 ± 0.0067 ^a	0.4455 ± 0.3309	<0.5	2
Stannum	0.1820 ± 0.0108 ^a	0.1420 ± 0.0125 ^b	0.1350 ± 0.0050 ^b	0.1373 ± 0.0067 ^b	0.1491 ± 0.0192	NA	NA
Copper	12.3497 ± 0.1476	12.9200 ± 0.6600 ^{ab}	12.1920 ± 0.3290 ^c	13.6067 ± 0.1106 ^a	12.7671 ± 0.5553	NA	NA
Chromium	1.4043 ± 0.0689 ^a	1.0287 ± 0.0206 ^b	1.3550 ± 0.1429 ^a	1.3037 ± 0.0227 ^a	1.2729 ± 0.1454	NA	NA

Data are presented as mean ± standard deviation of triplicate independent experiments. Mean values with different letters in the same row are statistically different ($P < 0.05$). "NA" = not available. ¹International bee pollen standards proposed by Campos et al. [33]; ²maximum accepted levels of metals according to the Malaysian Food Regulation, 1985 (Regulation 38), for food which is not specified.

4. Conclusions

Botanical origin and nutritional content of *H. itama* bee bread have been evaluated. *H. itama* bee bread is a good source of carbohydrates, proteins, minerals, and amino acids. Toxic heavy metals were detected but within the acceptable range. However, some of the values analyzed depend on the geographical locations. This study contributes towards the existing knowledge on the nutritional values, particularly of the stingless bee *H. itama* bee bread in Malaysia. It also provides insightful information to further develop bee bread as one of the functional foods in the current market. Quality standards for bee bread in Malaysia can be developed based on the data obtained in this study.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Amino acid chromatogram profile for samples from four different geographical locations (Batang Benar, MAEPS, Rinching Hilir, and Ladang 10 UPM). (*Supplementary Materials*)

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