Nutritional, Phytochemical and Antioxidant Analysis of Bee Bread from Different Regions of Malaysia

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Bee bread samples of stingless bee (*Heterotrigona itama*) collected from Kelantan (east coast), Selangor (central) and Perak (northern) regions of Malaysia were subjected to nutritional analysis using standard hydrolysis and oxidation methods. Phytochemical screening was carried out on aqueous and ethanol extracts of bee bread. Total phenolic and flavonoid contents, free radical scavenging activity and ferric reducing activity of the extracts were also assessed. All samples had essential and non-essential amino acids, carbohydrates, proteins, fat, flavonoids, tannins, phenols, xanthoproteins, cardiac glycosides, terpenoids, saponins, resins, and antioxidant activities. Sample from Kelantan had the significantly highest content of alanine, glycine and isoleucine, while sample from Selangor had the highest content of carbohydrate, total energy and proline. Sample from Perak had the highest content of fat. Bee bread ethanol extract demonstrated significantly higher antioxidant properties compared to bee bread aqueous extract with the highest total phenolic and flavonoid contents in sample from Perak and the highest free radical scavenging activity in sample from Selangor. Bee bread from Malaysia has good nutritional and antioxidant properties, which might indicate its potential to be a dietary supplement. The differences in compositions and antioxidant activities among the samples might be related to the floral sources and geographical locations.

Key words: Bee bread, extract, nutrients, amino acids, antioxidant

The consumption of bee products recently has been in high demand due to its powerful nutritional value and therapeutic properties. Bee bread has been included as one of scientific interest among honeybee's products. Bee bread is formed by bees which tightly pack the pollen into the beehives and store in the empty cells. Together with this, the bees collect the flower nectar and mix with its glandular secretion into the cells. About three quarter of the cell volume filled with the mixture and the remaining space is sealed with honey preparing for anaerobic environment inside. At this time, lactic acid fermentation takes place, subsequently providing bee bread rich in enzymatically-activated components[1,2].

Oxidative stress generates dangerous free radicals, which affect human health and exposing them to disease by altering the naturally structured cellular lining of human body subsequently causing lipid peroxidation and effects on enzyme activity as well as producing carcinogenesis. Antioxidants benefit human body by neutralizing and removing free radicals in the blood stream. Surprisingly, bee bread has been reported to possess antioxidant[3], antibacterial[4], anticancer[5] as well as hepatoprotective[6] properties. The presence of antioxidant properties in bee bread has been reported worldwide especially in Europe countries. Bee bread is regarded as a well-balance diet as it consisted carbohydrates, fat, proteins[7], fatty acids[8], and trace minerals[9] necessary for human health.

Composition of bee breads from various regions such as Lithuania[10], Araucania[11], Poland[7], Ukraine[1], Romania[9] and Georgia[3] have been studied. Romanian bee bread has been shown to have antioxidant properties by 1-1-diphenyl-2-picrylhydrazyl (DPPH) method.

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Phenolic content of bee bread has been quantified in methanol and aqueous extracts using Folin-Ciocalteu (FC) method[11]. Meanwhile flavonoid content was demonstrated in Georgian bee bread using high performance liquid chromatography[3]. Compositions of bee bread varies depending on its botanical origin and season[12]. However, to date, no study has been reported on detailed properties of bee bread from Malaysia. Therefore, this study was carried out to evaluate and compare the nutritional, phytochemical and antioxidant properties of bee bread from different regions in Malaysia.

Bee breads samples from stingless bee (*Heterotrigona itama*) were purchased from three different regions in Malaysia including Kelantan (east coast), Selangor (central) and Perak (northern), during the period January-June 2016. These regions were selected based on the suggestion by Malaysian local beekeepers who recognized the geographical advantages of these 3 regions where the bee bread domestic production were most concentrated. All bee breads were freshly received and dried using a food dehydrator at 35° until constant weight was achieved. Then each sample was ground to powder using a mini-blender and stored in a sterilized container at –20° until further analysis.

Analyses of carbohydrates, fats, and protein were carried out in accordance with in house method referred to Association of Official Analysis Chemists. Protein content was analysed using the Kjehdal method[13], which involved heating of total nitrogen with sulphuric acid up to boiling point. The oxidation caused the nitrogen to be converted to ammonium sulphate and it was estimated by titration[14]. The fat was extracted from the sample via hydrolysis followed by evaporation and condensation using a Soxhlet apparatus[14]. The difference of weight before and after the process was calculated and expressed as a percentage. Total carbohydrate was measured by the difference from total sum of protein and fat in percentage[14]. Meanwhile, energy (kcal) was estimated by using the conversion factors where, energy (kcal) = 4 (% carbohydrate)+4 (% protein)+9 (% fat).

Essential and non-essential amino acids were determined by Accq Tag Waters method[15]. It consisted of hydrolysis and heating process by using alpha-aminobutyric acid as an internal standard. The actual weight for each amino acid was recorded after the injection vial by using limited volume inserts. All of the results were carried out in duplicate and reported as percentage and standard deviation.

Bee bread aqueous (BBA) extract and bee bread ethanol (BBE) extracts were prepared using distilled water and 70 % ethanol, respectively. Briefly, 50 g of powdered bee bread was diluted in 10 volumes of distilled water or 70 % ethanol and was kept in dark place at room temperature for 72 h. After maceration, each solution was mixed with a magnetic stir at 500 rpm for 10 min followed by centrifugation at 4000 rpm for 10 min. Supernatant was collected, filtered via Whatman paper No. 1 and freeze-dried (5 mmHg pressure and –50°). The yields obtained as BBA and BBE extracts were kept in –20° refrigerator for phytochemical screening and antioxidant analyses which were done within one week.

Phytochemical screening was performed using standard methods to detect the presence of alkaloids, flavonoids, glycosides, phenols, resins, saponins, tannins, terpenoids and xanthoproteins. Fifty milligrams of each extract was added to 2 % hydrochloric acid (HCl) and Mayer's reagent. Appearances of cream coloured precipitate indicated the presence of alkaloids[16]. Two hundred milligram of each extract was mixed with 1 ml of distilled water or 70 % ethanol and one drop of 10 % (w/v) ferric chloride was added to the mixture. Formation of a brownish precipitate revealed the presence of flavonoids[17].

One hundred milligram of each extract was dissolved in 1 ml of glacial acetic acid containing one drop of 10 % (w/v) ferric chloride. Glycosides were identified by adding 1 ml of 98 % (v/v) sulphuric acid. Appearance of reddish brown-coloured ring at the interface of the mixture indicated for the presence of glycosides[18]. Fifty milligram of each extract was mixed with 5 ml of distilled water or 70 % ethanol. Appearance of intense purple colour indicated the presence of phenols[19]. Five hundred milligram of each extract was added with 5 ml of distilled water or 70 % ethanol. Appearance of turbidity indicated the presence of resins[18]. Five hundred milligram of each extract was shaken vigorously in 5 ml of distilled water or 70 % ethanol. The frothing solution was mixed with 3 drops of olive oil and then shaken. Appearance of persistent froth and emulsion upon mixing of frothing solution with oil indicated the presence of saponins[20].

Five hundred milligram of each extract was mixed with 20 ml of distilled water or 70 % ethanol. The mixture was then added with 2 ml of 0.1 % (w/v) ferric chloride. The appearance of olive green colour of mixture indicated the presence of tannins[21]. Five

hundred milligram of each extract was added with 2 ml of chloroform. Then, 3 ml of 98 % (v/v) sulphuric acid was carefully added to form a layer. Appearance of reddish brown colour indicated the presence of terpenoids[19]. Three drops of 15 % (v/v) nitric acid was added to 200 mg of each extract in 1 ml of distilled water or 70 % ethanol. Appearance of yellow colour of the mixture indicated the presence of xanthoproteins[18].

DPPH radical scavenging activity was evaluated using a previously reported method[22] with slight modification. Presence of antioxidant turned the purple colour of DPPH into yellow. The assay mixture contained 1.5 ml of DPPH radical solution (0.09 mg/ml in methanol) and 0.75 ml bee bread extracts. The absorbance of the remaining DPPH was determined after 15 min by spectrophotometer at 517 nm. The measurement was performed in triplicate. Radical scavenging activity was calculated by the formula below, I = [(Ab–Aa/Ab)]×100, where, I is % DPPH inhibition, Ab is the absorption of a blank sample and Aa is the absorption of bee bread extracts solution.

The antioxidant activity of bee bread was determined using ferric reducing antioxidant power (FRAP) assay according to the method reported earlier[23]. Briefly, 1.5 ml of freshly prepared FRAP reagent was added to 200 µl of each bee bread extract. Subsequently, the mixture was incubated at 37° for 4 min before the absorbance measured spectrophotometrically at 593 nm against a reagent blank. FRAP was prepared by mixing reagent of acetate buffer, 2,4,6-tris(2-pyridyl)-1,3,5-triazine solution in HCl and ferric chloride solution. Calibration curve of aqueous solution of ferrous sulphate heptahydrate was conducted with R^2: 0.9816. The test was performed in triplicate for each bee bread sample and result was expressed as millimole of ferrous per litre bee bread (mmolFe^2/l).

The content of total polyphenols was quantified according to the FC spectrophotometric method[24] using gallic acid as a reference standard. One millilitre of FC reagent (1:10) was added to 200 µl of bee bread extract and then was vortexed for 3 min. After the addition of 1 ml of 10 % sodium carbonate solution, the extracts were incubated for 90 min at room temperature in a dark place. Standard curve was constructed using gallic acid ranging from 20-100 µg/ml with R^2=0.9819. The test was carried out in triplicate and result was expressed as mg gallic acid equivalent/g bee bread (mgGAE/g).

Flavonoid contents were determined using aluminium chloride ($AICl_3$) reaction according to previous study[25]. Briefly, 4 ml of distilled water was added to 1 ml of bee bread extracts. At zero time, 0.3 ml of sodium nitrite (5 % w/v) was added followed by 0.3 ml of $AlCl_3$ (10 % w/v) after 5 min. Subsequently, 2 ml of 1 M sodium hydroxide solution was added after 6 min. The mixture was shaken vigorously after which the volume was made up to 10 ml of distilled water. Test was performed in triplicate by spectrophotometer at absorbance of 510 nm. Calibration curve of quercetin was prepared as standard with R^2: 0.9741. Results were expressed as mg of quercetin/g bee bread (mgQE/g).

All of the data were analysed using IBM SPSS version 22. Numerical data with normal distribution and homogenous variance were analysed using One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparison between all the three bee bread samples. Meanwhile, student t-test was used to compare the mean between ethanol and aqueous extract for each bee bread sample. The data were presented as mean (standard deviation) and a value of $p<0.05$ was defined as statistically significant.

To date, there are still not enough literature reporting the antioxidant activity and profile of bioactive compounds of bee bread specifically in Asian countries. Seventy percent of ethanol extract was used in this study as it is widely used due to its higher polarity compared to pure ethanol[26,27] and it can produce higher extraction yield[28]. This is the first study on nutrient analysis, amino acid content, and phytochemical screening of Malaysian bee bread.

Highest carbohydrate (59.55 %), total protein (18.37 %) and total energy (1478 kJ) contents were found in bee bread from Selangor (Table 1). Bee bread from Poland has higher carbohydrate content (71.9 %) as the major component constituted by monosaccharides[7]. Present study showed that bee bread from Perak had the highest content of fat. Analysis of Lithuanian bee bread indicated the presence of saturated and unsaturated long chain fatty acids, including omega-3 linolenic acid (ALA)[8]. All bee bread samples in the present study had both essential and non-essential amino acids (Table 2). Bee bread from Kelantan possessed significantly highest content of alanine, glycine and isoleucine. In other study, amino acids such as leucine, proline and valine were identified by gas chromatography-mass spectrometry method in small quantity in Romanian bee bread[7].

The phytochemical screening tests are helpful to determine the presence of bioactive components of an extract. Table 3 shows the results obtained from the phytochemical screening analysis of different bee bread samples in the present study. All nine phytochemical compounds were present in all BBE extracts while only alkaloids were not present in all BBA extracts. It was shown that the colour intensity for flavonoids, saponins and terpenoids was more in BBE extracts compared to BBA extracts indicating the higher concentration of these compounds in BBE extracts. This is due to their higher solubility in 70 % ethanol than in distilled water. Flavonoids and saponins exhibited higher concentration in BBE extract from Selangor while alkaloids and terpenoids exhibited higher concentration in BBE extract from Perak.

TABLE 1: NUTRIENT COMPOSITION OF DIFFERENT MALAYSIAN BEE BREADS

Nutrient component g/100 g	Bee Bread		
	Kelantan	Selangor	Perak
Carbohydrate	58.73 (0.66)	59.55 (0.34)	32.74 (1.74)[a,b]
Protein	17.64 (0.44)	18.37 (0.22)	17.22 (0.81)
Fat	2.17 (0.13)	4.51 (0.71)[a]	4.80 (0.2)[a,b]
Energy Kcal/100g	1365 (0.0)	1478 (0.0)[a]	1020.5 (17.68)[a,b]

Energy was calculated by summation of (fat×9 kcal)+(protein×4 kcal)+(carbohydrate×4 kcal). [a]p<0.05 compared with Kelantan group, [b]p<0.05 compared with Selangor group. One-way ANOVA followed by Tukey's post hoc test. All values are shown in mean (standard deviation)

TABLE 2: AMINO ACID CONTENT OF DIFFERENT MALAYSIAN BEE BREADS

Bee bread g/100 g	Kelantan	Selangor	Perak
Alanine	0.91 (0.00)	0.86 (0.01)[a]	0.89 (0.01)[a,b]
Arginine	0.90 (0.02)	0.77 (0.12)	0.74 (0.00)
Aspartic acid	1.50 (0.01)	1.47 (0.12)	1.45 (0.01)
Glutamic acid	2.10 (0.01)	2.08 (0.20)	2.00 (0.02)
Glycine	0.77 (0.00)	0.66 (0.08)[a]	0.68 (0.01)[a]
Histidine	0.49 (0.00)	0.35 (0.09)	0.37 (0.00)
Hydroxyproline	0.13 (0.02)	0.2 (0.06)	0.14 (0.00)
Isoleucine	0.74 (0.02)	0.65 (0.02)[a]	0.68 (0.00)
Leucine	1.20 (0.00)	1.06 (0.87)	1.14 (0.01)
Lysine	0.93 (0.01)	0.85 (0.15)	0.93 (0.02)
Methionine	0.39 (0.00)	0.31 (0.06)	0.34 (0.00)
Phenylalanine	0.77 (0.01)	0.67 (0.21)	0.65 (0.00)
Proline	0.83 (0.02)	1.56 (0.26)[a]	0.92 (0.00)[b]
Serine	0.81 (0.01)	0.74 (0.05)	0.74 (0.00)
Threonine	0.71 (0.01)	0.63 (0.09)	0.63 (0.00)
Thyrosine	0.48 (0.01)	0.42 (0.14)	0.35 (0.00)
Valine	0.84 (0.00)	0.78 (0.03)	0.81 (0.00)

[a]p<0.05 compared with Kelantan group, [b]p<0.05 compared with Selangor group. One-way ANOVA followed by Tukey's post hoc test. All values are shown in mean (standard deviation)

DPPH values of BBA extracts ranged from 2.86 to 8.95 %, in which significant highest content was found in bee bread from Kelantan (8.95 %). The DPPH scavenging activity of BBE extracts ranged from 72.04 to 79.34 % and highest activity was detected in a sample from Selangor (79.34 %) followed by samples from Kelantan (78.45 %) and Perak (72.04 %). Meanwhile, Romanian BBE extract had higher DPPH (89.45 %) compared to the samples from Malaysia in the present study. This wide range of results might be due to high polarity of 70 % ethanol compared to aqueous to dissolve the phytochemical compounds with antioxidant property[26].

Total phenolic content in BBA extracts ranged from 14.19 to 15.38 mg GAE/g, which significantly higher level was found in BBA extract from Selangor. These results are higher in comparison with Romanian BBA extract[29], which has total phenolic content of 8.32 mg GAE/g. In the present study, total phenolic content of BBE extract from Perak was significantly higher (22.54 mg GAE/g) than those samples from Selangor (21.96 mg GAE/g) and Kelantan (21.32 mg GAE/g), respectively. Moreover, it is noted that Malaysian BBE extracts had higher total phenolic content than

TABLE 3: PHYTOCHEMICAL SCREENING

Test	BBA extract			BBE extract		
	Kelantan	Selangor	Perak	Kelantan	Selangor	Perak
Alkaloids	-	-	-	+	+	+++
Flavonoids	++	++	+	++	+++	+
Glycosides	+	+	++	+	+	++
Phenols	++	+	+	+	++	++
Resins	++	++	+	++	++	+
Saponins	++	++	+	++	+++	+
Tannins	+	++	+	+	+	+
Terpenoids	++	+	+	++	+	+++
Xanthoproteins	+	++	+	++	+	+

BBA, bee bread aqueous; BBE, bee bread ethanol. A positive sign (+) indicates the presence of compound while double (++) and triple (+++) positive signs indicates the presence of compound with higher intensity of colour change

TABLE 4: ANTIOXIDANT ANALYSIS OF MALAYSIAN BEE BREADS

Antioxidant test	Bee Bread		
	Kelantan	Selangor	Perak
DPPH assay (%)			
BBA	8.95 (0.32)	3.24 (0.22)[a]	2.86 (0.40)[a]
BBE	78.45 (1.16)[*]	79.34 (1.46)[*]	72.04 (4.13)[b*]
FRAP assay (mmol Fe^2/l)			
BBA	0.94 (0.03)	1.00 (0.00)[a]	0.99 (0.01)[a]
BBE	1.07 (0.02)	1.08 (0.01)	1.08 (0.01)
Total flavonoid content			
(mg QE/g)	3.92 (0.09)	2.88 (0.28)[a]	3.77 (0.09)[b]
BBA	16.71 (0.57)[*]	16.48 (0.31)[*]	26.57 (0.83) [a,b*]
BBE			
Total phenolic content			
(mg GAE/g)	14.19 (0.18)	15.38 (0.37)[a]	14.35 (0.23)[b]
BBA	21.32 (0.16)[*]	21.96 (0.26)[*]	22.54 (0.11) [a,b*]
BBE			

BBA, bee bread aqueous extract; BBE, bee bread ethanol extract. DPPH; 1-1-diphenyl-2-picrylhydrazyl; FRAP, ferric reducing antioxidant power. [a]p<0.05 compared with Kelantan group, [b]p<0.05 compared with Selangor group. One-way ANOVA followed by Tukey's post hoc test. *P<0.05 compared BBA (Independent t- test). All values are shown in mean (standard deviation)

Romanian (13.92 mg GAE/g) and Ukraine (12.36 mg GAE/g)[1].

Similarly, total flavonoid content of BBE extracts in all regions was significantly higher than BBA extracts (Table 4). Our finding on total flavonoid content in Malaysian BBE extract was higher than Romanian extracts (5.13 mg QE/g)[30]. Meanwhile, BBA extract from Selangor possessed significantly highest FRAP value, whereas, insignificant differences of FRAP values were found for BBE extracts.

Taken together, BBE extracts showed higher total phenolic and flavonoid contents as well as higher antioxidant activities from DPPH and FRAP assays compared to BBA extracts which is in line with previous study[29]. These differences have been suggested to be attributed to the types of solvent used for the extraction, time interval of maceration as well as solvent concentration[31]. Furthermore, different solvents may have different polarity which can solubilise different phytochemical compounds with different chemical and biological properties[26]. Furthermore, the different findings between all the three samples could be attributed to the different floral sources in the different regions which was further supported by a previous study[12].

In conclusion, Malaysian bee bread has good nutritional and antioxidant properties, which might suggest its use as a dietary supplement. The differences of the compositions and antioxidant activities among the samples might be related to the stingless bee foraging activity on different floral sources in different geographical locations.

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