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Original Article

Antioxidant and antimicrobial properties of honey, propolis and bee bread of stingless bee (*Geniotrigona thoracica*)

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Abstract

Stingless bee (*Geniotrigona thoracica*) honey is stored in cerumen pots and it has been made from wax combined with propolis. Stingless bee propolis is resin that is collected by foraging worker from plants while bee bread is the pollen that has been stored in wax combs by bee's colony. The aim of this study is to determine antioxidant properties of honey, propolis, and bee bread of stingless bee and their antimicrobial activity against *Staphylococcus spp.*, *Bacillus spp.*, *Listeria spp.*, *Pseudomonas spp.*, *Salmonella spp.*, and *Escherichia coli.* The physical properties of these samples were significantly different ($p<0.05$) on color and pH value. There were significant differences ($p<0.05$) on moisture, ash, protein, reducing sugar and vitamin C content among samples. Based on antioxidant properties, there were also significant difference ($p<0.05$) between the three different samples. Propolis showed the highest amount of antioxidant content compared to honey and bee bread. Propolis and honey shared the highest total phenolic content (TPC) level which was 5.86±0.01 mg GAE/100g for propolis and 5.47±0.26 mg GAE/100g for honey. On the other hand, DPPH radical scavenging analysis indicated that propolis had the highest value which was 88.8±0.43%, followed by bee bread (59.38±0.64) and honey (57.60±1.20). FRAP assay also showed the highest value in propolis (38.88±0.81 µmol/g). Honey, propolis and bee bread showed different effect of antimicrobial activity ($p<0.05$) against five bacteria including *Staphylococcus spp.*, *Bacillus spp.*, *Listeria spp.*, *Salmonella spp.*, and *Escherichia coli* except *Pseudomonas spp*. ($p>0.05$). This indicated that different product of stingless bee showed different beneficial properties.

Keywords: Honey, Propolis, Bee bread, Stingless bee, Antioxidants, Antimicrobial

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Introduction

Stingless bees are a group of eusocial insects belonging to five different genera including *Melipona*, *Trigona*, *Meliponula*, *Dectylurina* and *Lestrimelitta* (Heard, 1999). Stingless bees have populated in

tropical earth for over 65 million years which is longer than Apis, the stinging honey bees (David, 2006). In Malaysia, the number of stingless bee species varies between 17 to 32 species depending on the study areas (Norowi, 2010; Salim et al., 2012). *Trigona* is the largest genus of stingless bees found exclusively in the Neotropics easily cultivated and more resistant to disease than *Apis mellifera*. Besides, it also has more diverse components of phytochemical due to a diversity of flavor, color and higher yield of its raw propolis. Besides producing honey, this genera also produced propolis (Choudhari et al., 2012), bee bread and royal jelly. Propolis is a natural product produced by bees, resulting from the addition of mandibular secretions to resins collected from different plant parts (Campos et al, 2014). Bee bread is a fermented mixture of plant pollen, honey, and bee saliva that worker bees use as food for the larvae, and for young bees to produce royal jelly (Markiewicz-Żukowska et al., 2013). The beneficial effect of bee products for health depends upon their source due to the strong variability in the antioxidant power.

Antimicrobial activity was also detected in stingless bee honey (Irish et al., 2008). The study of antimicrobial activity of honey from stingless bee demonstrated that Australian stingless bee (*Trigona carbonaria*) honey has broad-spectrum antibacterial activity but limited antifungal activity (Boorn et al., 2010). Based on Boorn et al. (2010), significant differences in antimicrobial activity were found amongst the 11 stingless bee honey samples, and individual test organisms were also found to differ in susceptibility. Propolis was also found with antibacterial, antifungal and anti-inflammatory properties and been used by man for over 3000 years (Briggs 2007). Propolis has a very broad chemical diversity because its composition varies with the site of collection, plant materials and producing species of bee (Bankova et al., 2000; Liberio et al., 2011). On the other hand, bee bread is which is produced from fermentation of bee pollen are rich in carbohydrates, crude fibres, proteins and lipids at proportions ranging between 13% and 55%, 0.3% and 20%, 10% and 40%, 1% and 10%, respectively (Villanueva et al., 2002). Pollen is rich in carotenoids, flavonoids, phytosterols, polyphenols and other beneficial compounds.

Studies on antioxidant activity of honey, propolis and other products inside the stingless bee nest itself are limited (Pérez et al., 2013). Apart from that, the medicinal properties such as antioxidant and vitamin analysis of the stingless bee honey and hive products are little explored in Malaysia. Honey from tropical countries is less known about its antioxidant capacity and mechanism involved by each biochemical component (Chua et al, 2013). Studies on the physic-chemical characteristics of the stingless bee products hamper the definition of quality patterns and standards are still lacking (Almeida et al., 2013). So, this study was focused on determining the antioxidant properties of honey, propolis, and bee bread of stingless bee and its relation to their antimicrobial activity against *Staphylococcus spp., Bacillus spp., Listeria spp., Pseudomonas spp., Salmonella spp.*, and *Escherichia coli*.

Material and Methods

Sample collection

Samples of stingless bee honey, bee bread and propolis were obtained from Kampung Tempinis, Jabi, Terengganu, Malaysia. Samples were stored at -20˚C until analysis.

Ethanolic extraction of propolis

Ethanolic extraction of propolis was conducted according to method by Kalogeropoulos et al. (2009) with some modifications. The crude propolis was frozen at -20°C before being grounded in chilled grinder. Crude propolis (10 g) was extracted under stirring 10-fold volume of 70% of ethanol solution in tightly closed bottle for 3 days at ambient temperature and minimum light. The suspensions then were subsequently frozen at -20°C for 24 hours. Filtration was done to the extract using Whatman No. 1 to remove waxes and soluble substances. The solutions were dried with rotary evaporator (40°C).

Ethanolic extraction of bee bread

Dry samples of bee bread were crushed and 20g were extracted on shaker with 80g of 95% (v/v) ethanol for 12 hours. The top layers were decanted (Extract A) and the rest of the sediments was re-extracted in the shaker with 40g of 95% (v/v) ethanol (Extract B). Extract A and B were pooled together and centrifuged at 3,000 rpm for 30 minutes at 20°C. The extract was evaporated at 40°C in rotary evaporator. The obtained residue was weighed and stored at -20°C (Markiewicz-Żukowska et al., 2013).

Physical and chemical analysis

Colour

A colorimeter (Konica Minolta Calorimeter) was used to measure the color. Results was expressed in L* (lightness), a* (redness), and b* (yellowness). Instrumental color determination was made on three measurements in different areas of the surface of the bread. In this color system, L* represents lightness (L* = 0 is black and L* =100 is white) and a* and b* are the color coordinates representing chromaticity: +a* is red and –a* is green; +b* is yellow and –b* is blue.

pH value

pH of samples was determined by dissolving 10 g of samples in 75 ml of distilled water and measured with a pH meter (Hach Company, Loveland, CO, USA). This was repeated on three separate occasions and the mean and standard deviation were determined (Boorn et al., 2010).

Moisture content

Moisture content was determined by drying 10 g samples in oven-drying for overnight (105˚C) until the weight constant. Percentage of moisture content was calculated (Tee et al., 1996) using the following equation:

$$\% \text{ Dry content} = \frac{\text{Weight of dried sample}}{\text{Weight of sample}} \times 100$$

$$\% \text{ Moisture content} = 100 - \% \text{ dry content}$$

Ash content

Ash content of sample was determined by incinerating 10 g sample in muffle furnace (550˚C) and cooled in desiccator. The percentage of ash was calculated (Tee et al., 1996) based on the equation below:

The distribution of *V. parahaemolyticus* in the peeled blood cockles from five different supermarkets shown in Table 1.

$$\% \text{ Ash} = \frac{\text{Ash weight}}{\text{Sample weight}} \times 100$$

Protein content

Samples (1 g) were digested in digestion unit for 30 min. The digester was then distilled in distillation unit. Finally, it was titrated with 0.1N hydrochloric acid, HCl until the light green solution turned to blue or grey. Value of crude protein is calculated (AOAC, 2000), by using this formula:

$$\% \text{ N} = \frac{(\text{T-B}) \times \text{N} \times 14.0 \times 100}{\text{Weight of sample}}$$

T = Volume of sample titration
B = Volume of blank titration
N = Normality of HC;
% Protein = % N x F
F = Protein factor; 6.25

Vitamin C

Vitamin C was determined using spectrophotometric method. The fresh sample was homogenized with pestle using metaphosphoric acid (5% metaphosphoric acid in 10% acetic acid solution in water). The sample was filtered and treated with 85 % sulphuric acid solution and 2,4-dinitrophenylhydrazine and incubated at 60 minutes in water bath and be read at 520 nm in spectrophotometer (AOAC, 1998).

Reducing sugar

Dinitrosalicylic (DNS) acid reagent was made by dissolving 1 g of 3, 5-DNS acid and 30 g of sodium potassium tartrate tetrahydrate in 50 ml of distilled water. 20 ml volume of 2 N NaOH is then added slowly and the total volume made up to 100 ml with distilled water. Each sample was diluted 1 in 1000 in distilled water to a reducing sugar concentration of 0.2–1 mg ml−1. 1.5 ml volume of DNS reagent is added to 0.5 ml of diluted sample and incubated in a heating block at 110°C for 5 min. Sample was left cool immediately in iced water and optical densities are measured at 540 nm (Boorn et al, 2010).

Cultivation of biological materials

Three species of gram negative bacteria including Escherichia coli ATTC 11775 (*E.coli*), *Salmonella enterica* ATTC 14028 and *Pseudomonas aeruginosa* ATTC 10145 (*P. aeruginosa*) together with three species of gram positive including *Staphylococcus aureus* ATTC 33862, 25923 (*S. aureus*), *Listeria monocytogenes* (*L. monocytogenes*) and *Bacillus cereus* (*B. cereus*) were cultivated on nutrient agar.

Antimicrobial analysis

The pure colony bacteria that were sub-cultured in nutrient agar were inoculated into saline water and the concentration was compared with 0.5 McFarland standard. Bacteria were applied on the agar by using swab cotton. Holes were made in the media by using

sterile tips and loop (Sommeijer et al., 1995). The plates were incubated aerobically at 37˚C for 24 hours. The antimicrobial properties were determined based on the inhibition.

Total phenolic content

Samples (5 g) was diluted to 50 ml distilled water and filtered through Whatman No. 1 paper. Then, 0.5 ml of the solution was mixed with 2.5ml of 0.2N Folin-Ciocalteu reagent for 5 min and added with 2 ml of 75g/l sodium carbonate. The mixture was incubated at room temperature for 2 hours. The absorbance was measured at 760 nm (Meda et al., 2005) with spectrophotometer (Merck Milipore, United States) and gallic acid was used as the standard. Result was expressed as a gallic acid equivalent (GAE/g dry weight basis).

2,2-diphenyl-2 picrylhydazy (DPPH) method

Total of 1.5ml of DPPH solution (0.1mm, in 95% ethanol) was incubated with concentration of the sample extract (0.75-5.0 mg). Mixtures was shaken and incubated for 20 minutes at room temperature. Absorbance was measured at 517 nm (Aris et al., 2009).

$$\text{Scavenging effect (\%)} = \frac{\text{(Absorbance of sample at 517 nm)}}{\text{(Absorbance of control at 517 nm)}} \times 100$$

Ferric reducing antioxidant power (FRAP) method

FRAP reagent was prepared by mixing 2.5ml of 10mM TPTZ solution in 40mM HCL, 2.5 ml of 20mM FeCl3, and 25 ml of 0.3 M acetate buffer at pH 3.6. The FRAP reagent was prepared daily and warmed before used. Sample (1 g) was well dissolved in 10 ml of n-hexane-acetone mixture (6:4). The sample solution was filtered through Whatman No. 4 paper. Aliquot of 200 µL of samples was mixed with 1.8ml of FRAP reagent and the absorbance was measured at 593 nm after incubation for 10 minutes (Chua et al., 2013).

Statistical analysis

All the obtained data was analyzed with one-way analysis of variance (ANOVA) using Minitab software to see the interaction between the samples the properties ($p< 0.05$).

Results and Discussion

Physical and chemical properties of honey, propolis and bee bread

Table 1 shows the physical and chemical properties of honey, propolis and bee bread which are pH value, moisture content, color, ash content, protein content, reducing sugar and also the amount of vitamin C.

pH value

pH value is the measurement of acidity or alkalinity of a solution which also acting as a physico-chemical parameter that associated with the microbial development in any food. Table 2 showed all samples were in acidic range which indicated their stability towards microbial spoilage (Tobor-Kapłon et al., 2005). The acidic condition of all samples may result from the fermentation that occurs in the beehive (Menezes et al, 2012). However, pH value was found highest in propolis extract ($p<0.05$) which indicated the less acidic condition compared to other two samples. The possible reason may be related to the moisture content of the propolis which was the lowest (Table 2). Moisture content is one of the important factors that accelerate the fermentation process by bacteria and reduce the pH (Singh and Singh, 2018).

Moisture content

For moisture content, honey and bee bread was recorded as highest in Table 1 with 32.04±0.17% ($p<0.05$). Honey of stingless bee species has very high in moisture and fluidity compared to *Apis mellifera* honey due to the high hygroscopic characteristic of *Meliponinae* honey. This high hygroscopic behaviour is preserved even when the environment of the hive has low humidity and as mentioned above, higher in moisture content is one of the factors that enhancing the fermentation process to occur which can contribute to the lower pH in honey. Propolis was found having the lowest moisture content which was 10.16±0.31% as water is not its major component. Propolis majorly made from primary resins and vegetable balsams (50%), waxes (30%), essential oils (10%), and pollen (5%) (Pasupuleti et al., 2017).

Colour analysis

L* represents the lightness of color which by mean the higher the L* value, the brighter the color of the samples. According to Table 1, honey had brighter colour compared to propolis and bee bread. Generally, dark colored honey has more minerals than light colored honey as proven during previous study whereas darker honeys may have four to six times more minerals than light colored honeys, especially manganese, potassium, sodium and iron (Nascimento et al., 2015). The similar theories can also be applied to the propolis (Table 1). It varies from dark-brown to reddish-brown, with a greenish tone (Ramos and Miranda, 2007) which may represent the higher flavonoid content (Woo, 2004)

Meanwhile, a* was its position between red and green (or redness). The green components (negative a* values) were present in the honey samples ($p<0.05$). The color of honey might be contributed by pigments such as chlorophylls, carotenoids, flavonoids and derivatives of tannins and polyphenols (A-Rahaman, 2013). b* its position between yellow and blue (or yellowness) (Ghaitaranpour et al., 2013). Table 1 clearly showed that the yellowness of bee bread (b*) is highest compared to other samples is probably due to the pollen colour. According to Gilliam (1979), bee bread or also known as fermented bee pollen are biochemically similar to the origin pollen but with higher nutrients due to the fermentation. However, the yellow colour may also represent the carotenoid content. Carotenoid has positive relation with yellow colour and also considered as yellow plant pigment (Liu et al. 2015). This is supported with study by Barene et al. (2015) which found 6.7 – 9.3 mg/100g of carotenoids in yellow colour of bee bread derived from Latvia.

Ash

Ash content represent the total amount of mineral in food (Ooi et al. 2012) which clearly shown highest in bee bread ($p<0.05$; Table 1). The high mineral content may results from the fermentation process of bee bread in the hive. Fermentation of bee bread produced high digestible minerals, amino acids, fats and sugars (Kieliszek et al. 2017). These important nutrients make the bee bread become the ready-to-eat food for the young bees (Urcan et al. 2018).

Protein

Bee bread contained the highest content of protein which was 13.83±1.01% among the samples ($p<0.05$) which may consist of 4-10% of essential amino acids such as methionine, lysine, threonine, histidine, leucine, isoleucine, valine, phenylalanine, and tryptophan (Komosinska-Vassev et al., 2015). Bee bread was a part of the pollen which supplies the remaining dietary requirements such as protein, lipids, vitamins, and minerals (DeGrandi-Hoffman et al., 2013). Markiewicz-Żukowska (2013) also reported that bee bread is the main source of proteins with essential amino acids, fats, minerals, vitamins, and flavonoids especially to the young bees.

Table-1: Physical and chemical properties of honey, propolis and bee bread.

Properties		Sample		
		Honey	Propolis	Bee bread
pH		3.03±0.01^{c}	5.67±0.05^{a}	4.64±0.04^{b}
Moisture content (%)		32.04±0.17^{a}	10.16±0.31^{c}	30.61±0.33^{b}
Color	L*	84.510^{a}	50.363^{c}	63.943^{b}
	a*	-4.043^{b}	1.287^{a}	-8.480^{c}
	b*	10.930^{c}	31.503^{b}	38.063^{a}
Ash content (%)		0.30±0.06^{c}	3.58±0.95^{b}	10.51±0.27^{a}
Protein content (%)		0.35±0.09^{c}	3.240±0.23^{b}	13.83±1.01^{a}
Reducing sugar (%)		28.24±0.24^{a}	16.63±0.33^{b}	15.80±0.37^{c}
Vitamin C (mg/100g)		84.5^{c}	169.0^{b}	422.5^{a}

Note: Different letter indicates significant different ($p<0.05$) between column.

Reducing sugar

Based on the table 2, the reducing sugar content was found highest in honey samples with the mean value of 28.24±0.24 m of absorbance ($p<0.05$) which normally lower than European bee honey (Amin et al., 2018). The higher amount of reducing sugars in honey compared to other samples is because sugar is the major compound in honey compared to bee bread and propolis (Amin et al., 2018). The reducing sugars in honey probably consist of glucose, fructose and sucrose but no maltose (Amin et al., 2018) while bee bread consist only fructose and glucose (Komosinska-Vassev et al., 2015).

Vitamin C

Table 1 showed the extremely highest of vitamin C in bee bread ($p < 0.05$) with the value of 422.5±0 mg/100g. Bee bread is a combination of honey, pollen and bee secretion (Bakour et al. 2017). Honey has its own vitamin C and pollen was also reported with variety of vitamins includes vitamin C (Herbert et al. 1985). In addition to that, Herbert et al. (1985) also reported that bee can synthesize vitamins in their gut from symbiotic organism. The combination of these elements then was fermented in the hive to produce other nutrients and also vitamins. Thus, these complete processes make the bee bread rich with beneficial nutrients such as vitamin C.

Antioxidant properties of honey, propolis and bee bread

Antioxidant properties which consist of total phenolic content (TPC), free radical diphenylpicrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assay was dominated by propolis (Table 2). Propolis has similar phenolic content with honey ($p>0.05$) and highest in both DPPH and FRAP assay ($p<0.05$). [Table 2]

Table-2: Antioxidant properties of honey, propolis and bee bread.

Antioxidant properties	Sample		
	Honey	Propolis	Bee bread
TPC (mg GAE/100g)	5.86±0.01^{a}	5.47±0.26^{a}	2.23±0.24^{b}
DPPH (%)	57.60±1.20^{b}	88.8±0.43^{a}	59.38±0.64^{b}
FRAP (µmol/g)	27.18±4.41^{b}	38.88±0.81^{a}	15.18±0.06^{c}

Note: Each value is presented as mean ± standard deviation (n=3). Different letter indicates significant different ($p<0.05$) between column.

Propolis was reported higher with complex phenolic compound such as caffeic acid phenethyl ester and prenylflavanone group which are responsible for its activities (Kongkiatpaiboon et al., 2015). This also answers the reason of higher antioxidant activity in propolis. The antioxidant activity in propolis may possess antitumour and antihepatotoxic activities (Schmidt et al., 2014). The dark colour of propolis obtained in this study (Table 1) also can be used as an indicator to the high antioxidant properties in propolis (Woo 2004; Marshall et al., 2014). In this study, honey also contained high antioxidant properties. The DPPH scavenging activity is still in the range (23.81% to 100%). Honey samples contain abundant free radical scavengers, that able to reduce the imbalance between free radical production and antioxidant level (Chua et al., 2013).

Bee bread was found with lowest TPC ($p<0.05$) but high in DPPH activity (Table 1). This is contrary with Ghasemzadeh et al. (2010) who mentioned the antioxidant activity is found linearly proportional with phenolic contents. However, the presence of constituents other than the phenolic compounds such as vitamin C, E and carotenoids may influence the total antioxidant activity (Al-Mamary et al., 2002).

Antimicrobial activity

Table 3 shows the diameter of inhibition zone produced by honey, propolis and bee bread of stingless bee for six food pathogens. Interestingly, different types of bee products showed different respond against 5 different class of bacteria ($p<0.05$) except for *P. aeruginosa* where all the 3 bee products gave the similar high inhibition activity ($p>0.05$). Gram positive bacteria which has thicker peptidoglycan is normally resistance to any antimicrobial agent, but in this study, all gram-positive bacteria were inhibited especially by honey and propolis. [Table 3]

Table-3: Diameter of inhibition zone produced by honey, propolis and bee bread of stingless bee for different bacterial strains using agar well diffusion assays.

Sample	Zone of inhibition for different microorganisms (mm)					
	Gram positive bacteria			Gram negative bacteria		
	L.monocytogenes	*S. aureus*	*B. cereus*	*E.coli*	*Salmonella*	*P. aeruginosa*
Honey	164.44±24.55^{c}	194.44±21.86^{a}	147.78±14.60^{b}	144.44±25.55^{a}	123.33±12.25^{b}	158.89±32.57^{a}
Propolis	195.56±17.40^{b}	163.89±22.88^{b}	205.56±13.33^{a}	000.00±00.00^{b}	000.00±00.00^{c}	140.00±11.18^{a}
Bee bread	284.44±20.68^{a}	000.00±00.00^{c}	000.00±00.00^{c}	000.00±00.00^{b}	312.22±15.63^{a}	134.44±13.33^{a}

Note: Each value is presented as mean ± standard deviation (n=3). Different letter indicates significant different ($p<0.05$) between row.

Honey has the capability to inhibit all of the tested pathogens while propolis has the antimicrobial properties against all pathogens except *E. coli* and *Salmonella* (Table 3). In previous study (Campos et al. 2014), propolis was also found to inhibit gram positive bacteria such as *S. aureus* but not gram negative (*E. coli*). Surprisingly, although bee bread only inhibited half of the tested pathogens but it has the highest activity ($p<0.05$).

Stingless bee honey also was reported to have higher antimicrobial activity compared to honey bee honey (Ewnetu at al., 2013). The highest antimicrobial activity of stingless bee honey may relate with its low pH which unfavourable for most bacteria. Different with propolis which has higher pH value (Table 1), the antimicrobial activity was probably contributed by the antioxidant compounds in propolis (Table 2). The secondary bioactive compounds such as phenolic was reported by a lot of studies to have high antimicrobial activity (Bahri-Sahloul et al., 2014; Puupponen-Pimiä et al., 2001).

Abouda et al. (2011) also found the higher antimicrobial activity of bee bread against gram positive compared to the gram-negative bacteria which contrary with this current study. The different result is probably due to the lower pH of the bee bread derived from stingless bee which stressful for most gram-negative bacteria. Shenoy et al. (2012) mentioned that high sugar concentration, low pH, proteinaceous compounds, and other components in honey may all assist in antimicrobial activity.

Conclusion

In conclusion, honey, propolis and bee bread showed different antioxidant and antimicrobial properties ($p<0.05$). The difference was probably related with the different physico-chemical characteristic of every product. Thus, this study is another new approach to show that different product of stingless bee showed different beneficial properties.

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Contribution of Authors

Ahmad FT: Conceived idea, data interpretation and manuscript writing
Lani MN: Co-supervising the research in microbiological part
Nazari SA: Data collection and manuscript writing
Hajar NHM: Data collection and manuscript writing
Hassan KNAM: Statistical analysis
Razak SBA: Assisted in selection of research area and sample collection
Hassan Z: Data interpretation and manuscript approval

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