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Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak)

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ABSTRACT

Stingless honey bees form a large group of bees that lack of a sting and are found among Meliponinae species indigenous to various tropical and subtropical regions. They are able to produce “stingless bee honey” that contains divergent categories of phenolic and flavonoid compounds and have been associated with antioxidant and antibacterial activity. This study examines the physicochemical properties, antioxidant-activity and anti-microbial activity of stingless bee honey from Malaysia that was produced by *Geniotrigona thoracica, Heterotrigona itama* and *Heterotrigona erythrogastra*. The results show that *G. thoracica* honey has the highest concentration of the total phenolic context (99.04 ± 5.14 mg/ml) and the greatest reducing power (19.05 ± 0.79%), while flavonoids (17.67 ± 0.75 mg/ml), reducing power (18.10 ± 0.35%), DPPH (47.40 ± 3.18%) and FRAP (50.66 ± 5.77 mM of Fe^{2+}/100 g) of *H. itama* honey is significantly higher than those of the other honeys. In addition, *G. thoracica* honey has the highest antibacterial activity against *Staphylococcus xylosus* (2.10 ± 0.10 cm), which is Gram-positive bacterium, and against *Pseudomonas aeruginosa* (1.60 ± 0.10 cm) and *Vibrio parahaemolyticus* (2.03 ± 0.06 cm), which are Gram-negative bacteria. These results suggest that stingless bee honeys possess useful amounts of phenolic and flavonoid compounds that are able to act as natural anti-oxidants and also have significant anti-microbial activity.

Introduction

Stingless bees are a huge and diverse monophyletic group of native eusocial bees that are abundant in tropical and subtropical regions throughout the planet, including Australia, Africa, Southeast Asia, and tropical America. They have a sting that has undergone evolutionary decline and is unlikely to be able to cause harm or injury to a human. Stingless bees are among the most highly developed bees and have existed for > 90 million years. They belong to the order Hymenoptera and comprise the tribe Meliponini in the family Apidae (Chuttong et al., 2016a). Moreover, they are closely related to the eusocial honey bees, to bumblebees and to orchid bees (Bradbear, 2009). They are active all year round worldwide and are usually found in colonies under the earth, in a fissure in rock, inside a hole in a tree or within the bough of a tree. Around 500 different species of stingless bees have been discovered altogether and these include > 300 species in the Americas, 50 species in Africa, 60 species in Asia, 10 species in Australia and four species in Madagascar (Bradbear, 2009).

Stingless bees are able to produce “stingless bee honey”, which is a blonde sugary liquid with a glorious taste and aroma. It can be separated into different categories based on the honey's physical and chemical constituents, which are related to the physiology of production of the raw material, the territorial location of the floral source, the species of bee and the conditions of the ecosystem in which the bees live. Stingless bee honey consists mostly of carbohydrates, water, amino acids, vitamins and minerals (Chuttong et al., 2016b). Additionally, it also contains unique and distinct phenolic and flavonoid compounds that seem to play a critical role in its antibacterial, anti-inflammatory and antioxidant activities of the Western honey bee *Apis mellifera* in previous studied (Liu et al., 2013).

When compared to *Apis mellifera* which is the world superior in honey production, Stingless bees produce and keep still less honey on a per hive basis (Chuttong et al., 2016a). The limited stingless bee honey yield, particularly as an international commodity, results in a little

Abbreviations: DPPH-1, 1-diphenyl-2-picryl-hydrazyl; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; TSS, total soluble solids; FRAP, Ferric reducing antioxidant power; PMS, phenazine methosulfate; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium chloride; TPTZ, tripyridyl triazine; TSA, tryptic soy agar

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knowledge about antioxidant and antibacterial property of stingless bee honey. Therefore, this study is perhaps the first observation to study of its kind.

Honey is an energy rich medical product produced by different species of honey bees including stinged and stingless bees. It has had a valued place in traditional medicine since ancient times, with many functional applications, which effect from its chemical and physical composition. The ancient Egyptians, Chinese, Greeks, and Romans utilized honey cure their affliction as diseases of the intestine and skin wounds (Rao et al., 2016). Since a few decades ago, honey recently became the focus of attention by several research groups. The most remarkable disclosure was their antioxidant and antibacterial activity which has been revealed in several researches.

Free radicals are natural byproducts that are created by oxidation and include hydroxyl radicals, superoxide anion radicals and hydrogen peroxide. They are created by sunlight, pollution, stress, processed food and also environmental toxins. They may lead to chain reactions in the body and are able to destroy cells. Many diseases have been linked to a body's burden of free radicals, including heart disease, diabetes, cancers, aging and other events. Antioxidants are molecules that inhibit the oxidation of other molecules. They are able to donate an electron to the unpaired valence electron within a free radical, thus boosting the immune system and preventing cell damage.

Honey is a huge source of antioxidants. It can be helpful at preventing damage or injury of cells by acting as a natural antioxidant against such reactive oxygen species. Different varieties of honey from various countries and geographical regions exhibited different antioxidant properties. The identified mechanisms by which honey does this are associated with honey's chemical composition, specifically the presence of phenolic and flavonoid compounds to reduce oxidative reactions or free radicals within the food systems and human health (Gismondi et al., 2017; Di Marco et al., 2016). The potential of it depends on the number and arrangement of the hydroxyl groups in the molecules of interest. Alvarez-Suarez et al. (2010), documented that five monofloral Cuban types of honey show a high correlation between total phenolic content and the results of the ferric reducing antioxidant power assay. Furthermore, using three honey samples from Malaysia, Tualang, Gelam, and Acacia honey, Chua et al. (2013), found that total flavonoid content is well correlated with three antioxidant assays that use different mechanisms, namely free radical scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH), the ferric reducing antioxidant power assay and the β-carotene bleaching assay.

The antibacterial properties of honey have been well known in folk medicine for many years. These healing powers are directly due to the honey's chemical composition, including the presence of hydrogen peroxide, as well as other non-peroxide factors. There are many non-peroxide constituents that form part of the antibacterial activity of honey and these include phenolic compounds, flavonoids and a number of other components Zainol et al. (2013), and Weston et al. (1999), reported that the phenolic compounds present in Manuka honey as well as in Manuka nectar, pollen and propolis, form a powerful mixture that creates the non-peroxide antibacterial activity of New Zealand Manuka honey. Estevinho et al. (2008), found that phenolic compounds extracted from honey from Northeast Portugal have antibacterial activity against Gram-negative and Gram-positive bacteria.

There are > 30 species of stingless bees found in Borneo (Sarawak) and the properties of the honeys produced by these bees have not been explored. This study examines the physicochemical properties, the antioxidant-activity and the anti-microbial activity of stingless bee honey, where past research seem to have been minimal effort. Moreover, some species are commonly used in domesticated in the agro ecosystem for meliponiculture as pollinating agents for many important crops in Malaysia and the remainder are species, mainly for reasons of the environment tolerance group since they are present in most locations (Jaapar et al., 2016). Therefore, the basic aim of this research was to study antioxidant-activity and the anti-microbial activity of stingless bee honey produced by *Geniotrigona thoracica*, *Heterotrigona itama* and *Heterotrigona erythrogastra*, which are species of bee indigenous to Borneo (Sarawak).

Materials and methods

Honey samples

Honey samples from three species of stingless bee, namely *Geniotrigona thoracica, Heterotrigona itama* and *Heterotrigona erythrogastra*, were obtained by Kie-Yiong Wong in his bee farm from *BEE EXC SCI TECK SDN. BHD* in Sibu, Sarawak, Malaysia. The harvesting took in September 2016, honey pots were penetrated with a keen tool and laboured through syringe extraction from independent and cooperative honey pots (Chuttong et al., 2016a). All honey samples were two times diluted and filtered through a 0.2 μm fliter (Millipore) in the laboratory to eliminate contaminating micro-organisms. All samples were adjusted to 35% moisture content and were stored at 4 °C before being examined. All tests were carried out in triplicate ($n = 3$) and the outcome averaged to give the mean ± SD.

Determination of total soluble solids (TSS), protein content and total phenolic content

Measurement of the total soluble solids (TSS) present in the honey samples was carried out by refractometry. The protein content of the honey samples was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard (Liu et al., 2013). The total phenolic content of the honey samples was determined by the Folin–Ciocalteu colorimetric method (Liu et al., 2013). Briefly, 100 μl of honey sample was diluted in 5 ml of water, then 500 μl of Folin–Ciocalteu reagent was added and sample mixed for 3 min. After that, 1 ml of Na_2CO_3 solution (3.5%, *w/v*) was added and mixed in. After 1 h of incubation at room temperature, the absorbance was measured at 725 nm.

Determination of sugars

Honey sugar contents including fructose, glucose, maltose and sucrose were assayed according to the modified method of Chuttong et al. (2016a), by high performance liquid chromatography (HPLC) with refractive Index detector (RID). A 5% (*w/v*) solution of stingless bee honey in distilled water and filtered through 0.45 mum filter paper and injected into HPLC system (Milford, Massachusetts, United States), which was equipped with a waters 1525 Binary HPLC pump, 717 plus Auto samplers, Waters 2414 Refractive index detector coupled to a computer with Empower Build 1154 Software. For the determination of sugars an Supercosil LC-NH2 column (25 cm × 4.6 mm, 5 μm), mobile phase with HPLC acetonitrile/water (72,25) was used at a flow rate 1 ml/min, with an oven temperature of 40 °C.

Determination of total flavonoid content

The total flavonoid content of each honey sample was measured by the aluminum chloride colorimetric method. Quercetin (≥95% (HPLC), Sigma-Aldrich, St. Louis, MO) (0–100 μg/ml) was used as a reference material (Liu et al., 2008). First, 500 μl of the honey sample was mixed with 1.5 ml of 95% alcohol, 100 μl of 10% aluminum chloride hexahydrate, 100 μl of 1 M potassium acetate, and 2.8 ml of deionized water, then the mixture was incubated in the dark at room temperature for 30 min. Finally, the absorbance at 415 nm was measured.

Radical-scavenging effect on DPPH

The free radical scavenging activity of the three honeys on DPPH• was assessed using1, 1-diphenyl-2-picryl-hydrazyl by the method of Liu

et al. (2008), Ascorbic acid (0.1 and 1.0 mM) (Sigma-Aldrich, St. Louis, MO) was used the positive control. Initially, 300 µl of the honey sample was mixed with 300 µl of DPPH radical solution (1.0 mM) and 2.4 ml of ethanol (99%). The solution was shaken and left in the dark for 30 min. After centrifugation at 4500 rpm for 5 min, the absorbance of the solution was measured at 517 nm. The percentage of free radical scavenging activity that targeted DPPH was calculated as (%) = [1 − (absorbance of each honey sample at 517 nm)/(absorbance of the control at 517 nm)] × 100.

Radical-scavenging effect on superoxide radicals

The superoxide anion radicals scavenging activity of the three honeys was assayed by the method of Liu et al. (2008), Ascorbic acid (0.1 and 1.0 mM) (Sigma-Aldrich, St. Louis, MO) was used the positive control. First, 300 µl of the honey sample was mixed with 300 µl of phenazine methosulfate (PMS; 80 µM), 300 µl of reduced nicotinamide adenine dinucleotide (NADH; 624 µM), and 300 µl of nitroblue tetrazolium chloride (NBT; 200 µM). The solution was shaken and left to stand in the dark for 5 min, then the absorbance at 560 nm was measured. The scavenging activity of superoxide anion radical was calculated as (%) by following formula: [1 − (absorbance of each honey sample at 560 nm)/(absorbance of the control at 560 nm)] × 100.

Reducing power

The reducing power of each honey was determined using the method of Liu et al. (2008), with some minor modifications. Dibutyl hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO) was used as a reference material. Initially, 250 µl of each honey sample was mixed with 250 µl sodium phosphate buffer (0.2 M, pH 6.6) and 250 µl potassium ferricyanide; $K_3Fe(CN)_6$, 1%). Next the solution was incubated at 50 °C for 20 min and then allowed to cool down to room temperature for 5 min. Then, 250 µl of Thrichloroacetic acid (TCA; 1%) was added to the sample. After centrifugation at 8000 rpm for 5 min, 250 µl of the upper layer was mixed with 250 µl distilled water and 500 µl ferric chloride (0.1%) in the ratio of 1:1:2. The reaction solution was left in the dark for 10 min and finally the absorbance was measured at 700 nm.

Ferric reducing/antioxidant power assay

This method depends on the reduction of ferric tripyridyl triazine ($Fe(III)(TPTZ)_2$) to its ferrous form, which creates an intense blue color (Fe^{2+}-TPTZ) in the presence of antioxidants (Khalil et al., 2011). The FRAP of honey was measured according to the modified method of Katalinic et al. (2004). The FRAP reagent was prepared before the test by mixing 10 ml of acetate buffer (300 mM, pH 3.6) with 1 ml of TPTZ solution (10 mM in 40 mM HCl) and 1 ml of ferric chloride ($FeCl_{3.}6H_2O$, 20 mM). Then the mixture was vortex and incubated at 37 °C. At this point 6 µl of honey was mixed with 180 µl of FRAP reagent. The absorbance was then read at 600 nm using an aqueous solution of $FeSO_{4.}7H_2O$ (Sigma-Aldrich, St. Louis, MO) as a standard solution. The units used for the FRAP values is mM of ferrous equivalents/100 g of honey sample.

Antibacterial activity

The antibacterial activity of honey was evaluated by the filter paper disc diffusion method (Liu et al., 2013). Bacterial inocula were obtained from Professor Wu-jun Du, Department of Entomology, Chung Hsing University; four Gram-positive bacteria were used, namely *Staphylococcus aureus*, *S. intermedius B*, *S. xylosus*, *Streptococcus alactolyticus* as well as six Gram-negative bacteria were used, namely *Citrobacter koseri*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella cholerasuis*, and *Vibrio parahaemolyticus*. All bacterial strains are clinical isolates from veterinary hospital. The optical density (OD) of the bacterial inocula in tryptic soy broth were adjusted to 0.1 at 600 nm and then 0.2 ml of this suspension was spread onto tryptic soy agar (TSA) plates. Wells were cut into the agar using a sterile cork borer with 8 mm diameter. Next, 100 µl of each honey sample that had been diluted four fold with deionized distilled water were transferred into each corresponding well onto the TSA plates and then the plates were incubated at 37 °C for 24 h aerobically. The average diameter of triplicate inhibition zone for each honey was calculated in centimeters (cm).

Minimum Inhibitory Concentration determination (MIC)

Four Gram-positive bacteria; *S. aureus*, *S. intermedius B*, *S. xylosus*, *S. alactolyticus* and three Gram-negative bacteria; *C. koseri*, *E. coli*, *P. aeruginosa* were sub-cultured on TSA at 37 °C for 24 h. Inoculum were prepared by collecting three colonies from the overnight plates using a sterile inoculating wire loop and the collected bacteria was added to a sterile tube containing 10 ml of TS broth. The bacteria were then incubated at 37 °C overnight.

The MIC of honey was measured using a modification of the method of Zainol et al. (2013). The optical density (OD) of the bacteria suspension was adjusted using sterile TS broth to 0.5 McFarland (1 to 2×10^8 cfu/ml) at 625 nm (Clinical and Laboratory Standards Institute/NCCLS, 2012). The inoculum was then diluted by mixing 0.05 ml of the modified inocula with 9.95 ml of TS broth to give a bacterial cell density of 5×10^5 cfu/ml. A stock solution of each honey (50% *w*/w) was prepared by weighing out 5 g of each honey and adding this to 10 ml of TS broth. Serial dilutions were then prepared to give honey concentrations of 0.5%, 1%, 2%, 3%, 5%, 10%, 15%, 20%, 25%, and 50%. The final volume in each test well was 200 µl with three replicates per dilution, each consisting of 190 µl of each honey dilution and 10 µl bacteria inoculum. For each assay, various control wells were included: 1) wells containing 200 µl TS broth only (without honey and inoculum), this was used as assay broth sterility control; 2) wells containing 190 µl TS broth and 10 µl inoculum (without honey), which served as the viability control; and 3) wells containing 200 µl honey dilution with TS broth (without inoculum), which acted as dilution sterility controls. Incubation took place on 96 well flat-bottom microtitre plates in a shaker incubator at 120 rpm at 37 °C overnight and next day the absorbance of the wells was measured at 590 nm. The percentage inhibition of bacteria growth for each honey dilution was calculated by the following formula:

1 − (Absorbance of test well − Absorbance of dilution sterility control well)/(Absorbance of assay viability control − Absorbance of broth sterility control) × 100. The minimum value for the percent inhibition is 0% and maximum value is 100%.

Minimum Bactericidal Concentration determination (MBC)

Honey dilutions from the MIC test after the MIC assay were examined by the streak plate method. Honey dilutions from two test wells that presented with no growth of the test organism were randomly selected and the contents were streaked onto TSA using a wire loop. These plates were then incubated at 37 °C overnight aerobically. The minimal concentration of the diluted honey that showed no growth of the test inoculum ($\geq$1%) was considered to be the MBC (Zainol et al., 2013).

Statistical analysis

All experiments were carried out in triplicate and are presented as the mean ± standard deviation. Statistical analysis was carried out using one-way ANOVA and the Minitab 17.0 software for Windows. Tukey pairwise comparisons were used to detect differences between means. Differences between the means at the 95% ($p \leq 0.05$) confidence level were considered to be statistically significant.

Table 1
Chemical properties of the stingless bee honeys from Borneo (Sarawak).

Honey produced by	Phenolics (mg/ml)	Flavonoids (mg/ml)	Protein (mg/ml)	Total (Brix)	Fructose (%)	Glucose (%)	Maltose (%)	Sucrose (%)
Geniotrigona thoracica	99.04 ± 5.14[a]	14.97 ± 0.56[b]	3.12 ± 2.01[b]	65.0	12.01 ± 1.1	17.63 ± 1.5	35.3 ± 1.4	ND
Heterotrigona itama	67.86 ± 7.40[b]	17.67 ± 0.75[a]	3.59 ± 0.52[b]	75.2	19.5 ± 1.3	21.0 ± 1.7	33.7 ± 2.1	ND
Heterotrigona erythrogastra	44.72 ± 6.50[c]	12.41 ± 0.62[c]	5.66 ± 1.00[a]	73.0	12.3 ± 0.9	14.9 ± 0.7	45.2 ± 1.1	ND

Note: The data are expressed as mean ± S.D. ($n = 3$); the different letters within the rows indicate statistically significant differences determined using ANOVA ($p < 0.05$). ND: not detected.

Results and discussion

Physicochemical properties

Table 1 indicates the total phenolic content, flavonoid content, protein content and total soluble solid present in the three stingless bee honeys produced by three distinct bee species. The honeys from the different bee species had different physicochemical parameters. The study by Kek et al. (2014), showed that there are significant differences in terms of total phenolic content when honey samples classified as Malaysian honeys from *Apis* spp. and *Trigona* spp. are compared. Biluca et al. (2016), reported that the phenolic content of thirty three samples of honey from ten stingless bee species showed significant variability, with values ranging from 10.3 to 98.0 mg GAE 100 g^{-1}. In our study the phenolic content of the honey samples from the three stingless bee species varied from 44.72 ± 6.50 to 99.04 ± 5.14 (mg/ml). The honey produced by *G. thoracica* showed the highest phenolic content. The flavonoid compounds also present in honey were also responsible for some of the honeys' antioxidant activity (Nayik et al., 2016). The values for the flavonoid content of the three samples of stingless bee honey ranged from 12.41 ± 0.62 to 17.67 ± 0.75 (mg/ml) with $p \le 0.05$ between species. The honey produced by *H. itama* had the highest flavonoid contents. In terms of protein content, the honey made by *H. erythrogastra* had the greatest amount of protein 5.66 ± 1.00 (mg/ml). Moreover, the total soluble solid of the stingless honey samples in this study ranged from 65.0% to 75.2%, fructose content ranged from 12.01 ± 1.1% to 19.5 ± 1.3%, glucose content ranged from 14.9 ± 0.7% to 21.0 ± 1.7%, and maltose content ranged from 33.7 ± 2.1% to 45.2 ± 1.1%. When the physicochemical properties of the three Borneo (Sarawak) stingless bee honeys are compared to those produced by *A. mellifera* in Taiwan, Taiwan honey has a higher protein content 10.62 ± 0.95 (mg/ml), but has both a lower phenolic content (40.21 ± 5.65 mg/ml) and a lower flavonoid content (10.15 ± 0.61 mg/ml).

Antioxidant activity

Due to presence of phenolic acids and flavonoids compounds, honey is considered to be an abundant source of antioxidant activity (Nayik et al., 2016). The many and divergent varieties of honey found throughout the world show substantial levels of variation in their antioxidant activity. These honeys can be distinguished in terms of their physical and chemical constituents that lead to different levels of antioxidant activity and this variation may be due to the species of bee that produces the honey. The antioxidant activity levels of stingless bee honey from Borneo (Sarawak) were evaluated using a variety of approaches and the results are presented in Table 2.

DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) is used in a radical scavenging assay that is based on electron-transfer; it assesses the scavenging potential of a given substance (Garcia et al., 2012). The assay produces an intense violet solution that is stable at room temperature. When a DPPH solution is mixed with a test compound that is able to donate a hydrogen atom (Kedare and Singh, 2011), this changes the color of the compound to colorless or light yellow as the free radicals are scavenged. In the present study, the DPPH assay values for the three stingless bee honey from Borneo (Sarawak) ranged from 17.0 ± 7.5 to 47.4 ± 3.2 (%) and was similar with the positive control, ascorbic acid (Data not shown). The honey made by *H. itama* and *H. erythrogastra* had high DPPH radical-scavenging activity.

The reducing power assay is a method of assessing the capacity of an antioxidant to donate an electron. The presence of antioxidants in a test material results in the reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}) (Irshad et al., 2012). The reducing power of the stingless bee honey produced by the different bee species is presented in Table 2 and ranged from 11.86 ± 0.21 to 19.05 ± 0.79 (%) and was similar with the positive control, Dibutyl hydroxytoluene (BHT) (Data not shown).The honey produced by *G. thoracica* and *H. itama* were found to have significantly levels of higher reducing power than the honey produced by *H. erythrogastra* ($p \le 0.05$)..

Superoxide radicals are produced by PMS-NADH systems via the oxidation of NADH bring about the reduction of nitroblue tetrazolium (NBT) to purple formazan (Elmastas et al., 2003). Thus in the system used here, stingless bee honey samples that have superoxide radical scavenging activity are able to reduce the rate of the production of blue formazan (Zhang et al., 2012) which is measured via absorbance at 560 nm. Table 2 presents the percent inhibition of superoxide radical generation by the three stingless bee honey produced by different bee species. The stingless bee honey samples showed no significant differences in superoxide radical scavenging activity and the values ranging from 54.1 ± 6.9 to 61.4 ± 7.4 (%) and was similar with the positive control, ascorbic acid (Data not shown).

The Ferric Reducing Antioxidant Power assay (FRAP) measures the ability to convert ferric to ferrous ion, that is Fe(III) to Fe(II) (Benzie and Strain, 1996a, 1996b). This results in the formation of a colored ferrous-tripyridyltriazine complex. Reduction at a low pH causes a color change from colourless to blue, which can be measured via the absorbance at 600 nm. In the present study, the FRAP assay values for the three stingless honey samples had a relatively narrow range from 25.78 ± 4.80 to 50.66 ± 5.77 (mM of Fe^{2+}/kg) and was similar with the standard solution $FeSO_4.7H_2O$ (Data not shown) with the honey produced by *H. itama* having a markedly higher FRAP assay value compared to the other two honeys ($p \le 0.05$).

Chua et al. (2013), reported that both phenolic and flavonoid compounds in honey play important roles in antioxidant activity. The various antioxidant assay assesses the ability of these phenolic and flavonoid compounds to donate an electron from a hydroxyl group to unpaired electron of free radicals. In this study honey produced by *H. itama* has the highest phenolic content, while honey produced by *G. thoracica* has the highest flavonoid content (Table 1); both of these honeys have high reducing power (Table 2). Liu et al. (2013), showed that honey produced by *B. pilosa* had a higher phenolic and flavonoid content and this was also associated with a higher reducing power. Moreover, Attanzio et al. (2016), reported that monofloral honey produced by Sicilian black honeybees showed a correlation ($p < 0.0001$) between total phenolic content and parameters related to two activities, namely free radical scavenging activity via the DPPH assay ($r = 0.774$) and the FRAP assay ($r = 0.883$).

It has been suggested that the antioxidant capacity of honey could be the result of the combined activity of a narrow range of compounds (Sant'Ana et al., 2011), not only phenolic and flavonoid compounds, but

Table 2
Antioxidant activity of the stingless bee honeys from Borneo (Sarawak).

Honey produced by	[a]DPPH (%)	[b]Reducing Power (%)	[c]Superoxide Radical (%)	[d]FRAP (mM of Fe^{2+}/100 g)
Geniotrigona thoracica	17.07 ± 7.52[b]	19.05 ± 0.79[a]	54.13 ± 6.94[ns]	38.03 ± 2.27[b]
Heterotrigona itama	47.40 ± 3.18[a]	18.10 ± 0.35[a]	61.41 ± 7.42[ns]	50.66 ± 5.77[a]
Heterotrigona erythrogastra	44.56 ± 1.75[a]	11.86 ± 0.21[b]	58.09 ± 10.93[ns]	25.78 ± 4.80[c]

Note: The data are expressed as mean ± S.D. ($n = 3$); the different letters within the rows indicate statistically significant differences determined using ANOVA ($p < 0.05$). Ns: no significant difference.
[a] 1,1-diphenyl-2-picrylhydrazyl scavenging activity as a percentage (%).
[b] Reducing power as a percentage (%).
[c] Superoxide radical scavenging activity as a percentage (%).
[d] Ferric reducing/antioxidant power in mM of ferrous equivalents/100 g of honey sample.

also other components. Escuredo et al. (2013) found that there was correlation between protein content and the antioxidant activities of various honeys ($p < 0.01$). In this study, *H. erythrogastra* and *H. itama* had the highest DPPH radical-scavenging activity (Table 2), as well as having a high protein content and a high phenolic content, with mean values 5.66 ± 1.00 and 17.67 ± 0.75 (mg/ml), respectively (Table 1). When the antioxidant properties of honeys produced by *A. mellifera* in Taiwan are compared with our Borneo (Sarawak) stingless bee honeys, the Taiwan honeys show similar findings with a DPPH value of 41.4 ± 0.8 (%), but with high reducing power 19.5 ± 0.23 (%) and a lower FRAP value 13.01 ± 307.9 (mM of Fe^{2+}/100 g). They also showed similar results for the superoxide radical assay 64.8 ± 1.6 (%) (Data not shown). Therefore, it is possible that the protein content of *A. mellifera* honey from Taiwan plays a role in antioxidant activity of these honeys. In summary, the complex composition of honey and the various interactions between the different antioxidant components that are present are likely to be important to the overall antioxidant activity of honey; these factors include phenolic compounds, peptides, organic acids, enzymes, Millard reaction products and possibly other minor components (Gheldof et al., 2002).

Antibacterial activity

All three stingless bee honeys from Borneo (Sarawak) showed antibacterial activity against general animal pathogens both Gram-negative and Gram-positive microorganisms when tested by the filter paper disc diffusion method (Table 3). Furthermore, the honeys produced by *G. thoracica* and *H. erythrogastra* were able to inhibit the growth of all of the various bacterial species tested. By way of contrast, honey produced by *H. itama* showed no inhibitory activity against *K. pneumonia, S. cholerasuis*, and *V. parahaemolyticus,* which are Gram negative bacteria. Another notable point is that *G. thoracica* honey showed significantly greater inhibition of *S. xylosus*, *P. aeruginosa*, and *V. parahaemolyticus* than the other honeys ($p \leq 0.05$).

After the filter paper disc diffusion was tested were carried out, MIC and MBC assays were carried out using *S. aureus*, *S. intermedius B*, *S. xylosus*, *S. alactolyticus*, *C. koseri*, *E. coli*, and *P. aeruginosa.* The results are presented in Table 4. The honeys produced by *G. thoracica* had the lowest MICs at ≥5% (*w*/w) and the lowest MBCs at ≥10% (w/w). By way of contrast, the MIC and MBC of *H. itama* honey were ≥10% (w/w) and ≥20% (w/w), respectively. When compared to the *A. mellifera* honey standard from Taiwan, Taiwan honey had a higher MIC at ≥25% (w/w) and a higher MBC at ≥50% (w/w) than all three Borneo (Sarawak) stingless bee honey (data not shown). Fig. 1 presents the details of the growth inhibition experiments (%) using different concentrations of stingless bee honey (0–15% w/w) as MICs. At a concentration of ≥25% (w/w), all stingless bee honeys were active against all of the microorganism tested (data not show). The various bacteria showed different MIC_{50} patterns with the three honeys. The honey produced by *H. erythrogastra* showed the broadest activity against all of the bacterial strains tested. All three stingless bee honeys also show antifungal activity to against the fungus *Alternaria brassicae* at the high concentrations ≥ 60% (data not show).

The presence and activity of the antibacterial factors known to be present in honey varies widely and is likely to influence a given honey's

Table 3
Antibacterial activity of the stingless bee honeys from Borneo (Sarawak).

Bacteria strain/ honey type	Inhibition zone (cm)		
	Geniotrigona thoracica	*Heterotrigona itama*	*Heterotrigona erythrogastra*
Gram positive			
Staphylococcus aureus	1.70 ± 0.26[ns]	0.80 ± 0.70[ns]	1.50 ± 0.00[ns]
Staphylococcus intermedius B	1.67 ± 0.58[ns]	0.33 ± 0.58[ns]	1.00 ± 0.87[ns]
Staphylococcus xylosus	2.10 ± 0.10[a]	1.63 ± 0.12[b]	1.77 ± 0.66[b]
Streptococcus alactolyticus	2.0 ± 0.00[b]	2.5 ± 0.00[a]	1.53 ± 0.66[c]
Gram negative			
Citrobacter koseri	3.00 ± 0.10[ns]	3.30 ± 0.36[ns]	3.37 ± 0.06[ns]
Escherichia coli	1.83 ± 0.15[ns]	0.50 ± 0.87[ns]	1.00 ± 0.87[ns]
Klebsiella pneumoniae	1.23 ± 0.21[ns]	NI	1.30 ± 0.17[ns]
Pseudomonas aeruginosa	1.60 ± 0.10[a]	1.13 ± 0.12[b]	1.53 ± 0.15[a]
Salmonella cholerasuis	1.83 ± 0.29[ns]	NI	1.33 ± 0.29[ns]
Vibrio parahaemolyticus	2.03 ± 0.06[a]	NI	1.50 ± 0.10[b]

Note: The data are expressed as mean ± S.D. (n = 3); the different letters within the rows indicate statistically significant differences determined using ANOVA ($p < 0.05$). Ns: no significant difference, NI: no inhibitory activity.

Table 4
Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of the stingless bee honeys from Borneo (Sarawak).

Microorganism	Stingless bee honey produced by					
	Geniotrigona thoracica		*Heterotrigona itama*		*Heterotrigona erythrogastra*	
	MIC (% w/w)	MBC (% w/w)	MIC (% w/w)	MBC (% w/w)	MIC (% w/w)	MBC (% w/w)
Gram positive						
Staphylococcus aureus	5	10	10	15	3	15
Staphylococcus intermedius B	3	5	5	10	3	10
Staphylococcus xylosus	3	5	5	10	2	10
Streptococcus alactolyticus	5	10	10	20	5	15
Gram negative						
Citrobacter koseri	3	10	10	15	3	10
Escherichia coli	5	10	10	15	3	10
Pseudomonas aeruginosa	5	10	10	20	5	10

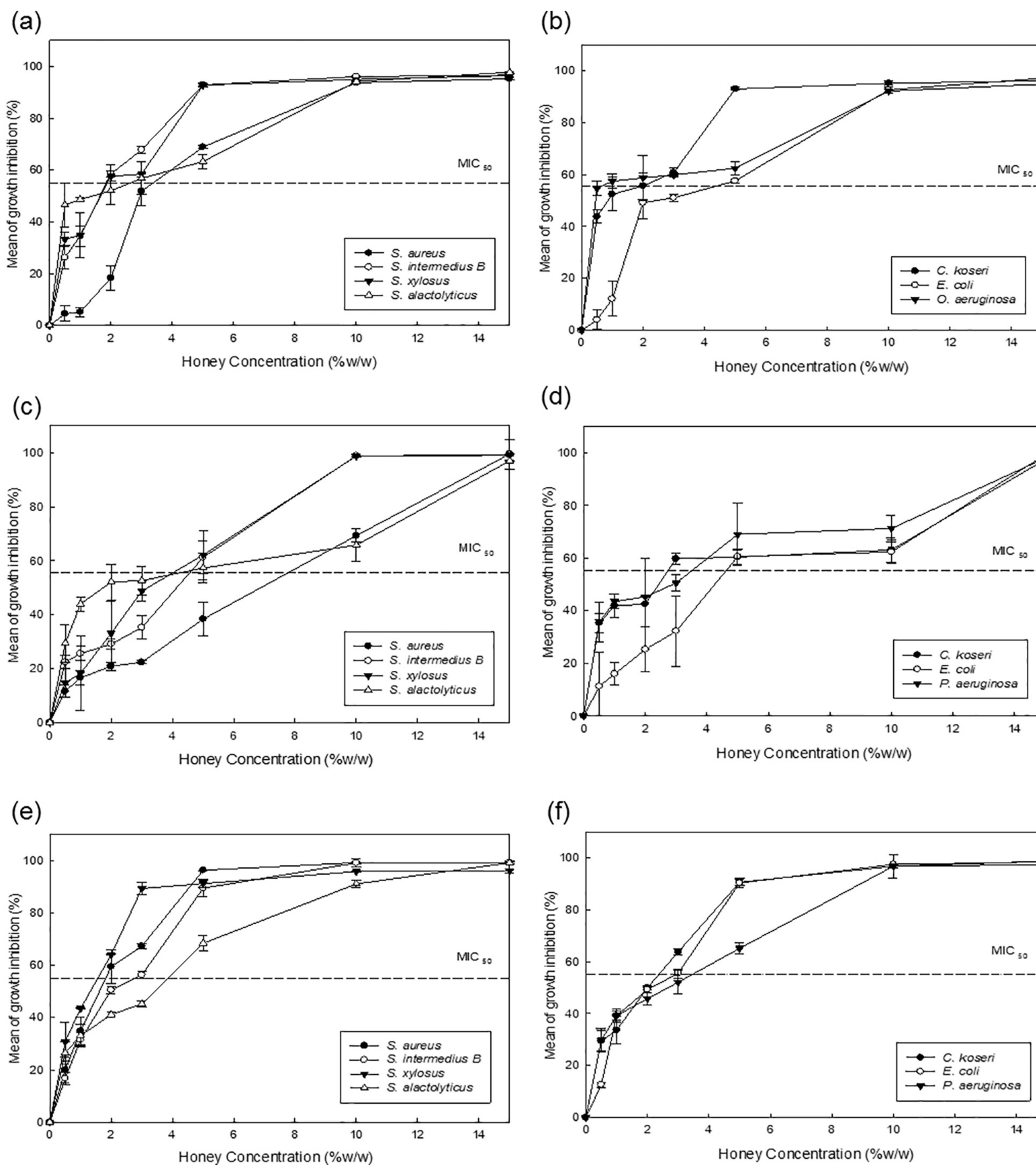

Fig. 1. Inhibition of microbial growth by stingless bee honey from Borneo (Sarawak) (a) *G. thoracica* honey tested against Gram-positive bacteria; (b) *G. thoracica* honey tested against Gram-negative bacteria; (c) *H. itama* honey tested against Gram-positive bacteria; (d) *H. itama* honey tested against Gram-negative bacteria; (e) *H. erythrogastra* honey tested against Gram-positive bacteria; (f) *H. erythrogastra* honey tested against Gram-negative bacteria.

overall effectiveness (Fyfe et al., 2017). It is well known that polyphenols, including phenolic and flavonoid compounds, can play a critical role in the non-peroxide antibacterial activity of honey. It is their chemical structure, namely the presence of benzene ring substitutions and saturated side-chain on the polyphenol that create the activity against bacteria. In addition, Souza et al. (2016), suggested that the presence of significant amounts of flavonoids in honey from Juazeiro, which is produced by a stingless bee, could also be involved in the powerful antibacterial activity of this honey.

The antibacterial activity of the various different polyphenols occurs in various way such as alterations in microbial cell permeability (Fyfe et al., 2017; Escuredo et al., 2012). However, Souza et al. (2016), reported that honey samples with the highest phenolic content had the smallest MICs against the bacterial strains used in their tests. These findings agree with the present study where honey produced by *H. itama*, which has the highest phenolic content, failed to inhibit some Gram-negative bacterial strains. On the other hand, the honey samples with the highest flavonoid content in our study showed significantly greater inhibition of the various Gram-negative bacteria tested. Similarly, Bueno-Costa et al. (2016), reported that there was no significant correlation between the total content of phenolic compounds (TCPC) in honey and their antibacterial activity against *Shigella dysenteria*, *S.*

typhimurium, S. aureus and *Bacillus cereus,* specifically, honeys from the Rio Grande do Sul, Brazil ($p > 0.05$). In the same study, these honey samples showed a correlation ($p \leq 0.05$) between total content of flavonoids and antibacterial activity against *B. cereus* ($r = 0.4424$). Similarly, Fyfe et al. (2017) in a study of Scottish honeys, found that there was no significant correlation between TPC and antibacterial activity against any of the bacterial strains tested.

From the chemical point of view, Flavonoids have antibacterial properties, due to disruption of membrane function or DNA synthesis. This may affect in the modulation of pathogenic microorganism composition. An independent antibacterial flavonoid has multiple cellular targets, rather than one specific site of action (Kumar and Pandey, 2013). The phenolic A- and B-rings of flavonoids generally contain hydroxy (OH) and/or methoxy (MeO) groups. These common structural characteristics may completely be necessary for flavonoids to intake into the bacterial cell. Mori et al. (1987) suggested that the B ring of the flavonoids may intercalate or form hydrogen bond with the stacking of nucleic acid bases and this may explain the inhibitory action on nucleic acid synthesis in bacteria. In an investigation into the antibacterial action of propolis, Mirzoeva et al. (1997) showed that one of its constituent flavonoids, quercetin, caused an increase in membrane permeability and a dissipation of the membrane potential which may decrease the resistance of cells to other antibacterial agents and contribute enormously to its overall antibacterial activity.

Furthermore, whether a bacterial strain is Gram-positive or Gram-negative does seem to have an effect on the antibacterial activity of honey. Using monofloral honeys from the central region of Cuba, Alvarez-Suarez et al. (2010), reported that *S. aureus,* which is Gram-positive, had high sensitivity to these honeys with a low MAD mean value (4.02% v/v). These results were compared to those obtained using a Gram negative bacteria *P. aeruginosa,* which had a much higher MAD mean value (11.86% v/v).

Overall, many factors seem to affect the antibacterial activity of the phenolic and flavonoid compounds present in honey, including the chemical structure of the compounds present in the honey and the bacteria strains used for testing. Nevertheless, it seems clear that the phenolic and flavonoid compounds found in stingless bee honey from Borneo (Sarawak) do have significant antibacterial activity.

Conclusion

Stingless bee honeys are considered to be an energy rich medicinal product and can be distinguished by their physical and chemical constituents. These differences are, at least to some extent, the result of these honeys being produced by unusual bee species. The results presented in this study demonstrated that *G. thoracica* honey has the highest flavonoid content, while *H. itama* honey has the highest phenolic content which was linked in the antioxidant activity of honey that can reduce oxidative reactions or free radicals within the food systems and human health. They can donate hydrogen atoms to free radicals which are more stable after becoming paired electron. Moreover, their chemical structure, namely the presence of benzene ring substitutions and saturated side-chain on the polyphenol, including phenolic and flavonoid compounds, can play a critical role in the non-peroxide antibacterial activity of honey, that create the activity against bacteria. Both of these groups of compounds play a key role in the various biological properties of these honeys, specifically anti-oxidant activity and anti-microbial activity.

Disclosure

The authors alone are responsible for the content and writing of the paper.

Conflicts of interest

The authors report no conflicts of interest.

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