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Physicochemical Analysis and Antioxidant Activity of Honey from Three Malaysian Stingless Bees Species

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ABSTRACT

This study investigates physicochemical and antioxidant activity of raw and honey extract from three different species of Malaysian stingless bees. The methanol extract of commonly found *Heterotrigona itama* (EHI), *Tetratgonula laeviceps* (ETL) and *Geniotrigona thoracica* (EGT) was tested for their total flavonoid, total phenolic, moisture, ash, fat, protein and carbohydrate. Antioxidant activity through DPPH radical scavenging activity was also determined. Nutritional analysis of raw honey showed a comparable result. Analysis of total flavonoid and total phenolic for honey extract showed a significant difference between three species. In contrast, raw honeys did not show any significant differences between species. The antioxidant testing showed that the three honeys does not possess good antioxidant activity with inhibition lower than 50%. ETL showed the highest inhibition with IC50 of 37.8%, followed by EHI with 24.3% and EGT with 9.8% of inhibition, respectively. Thin layer chromatography analysis revealed the content of phenolic and flavonoids in all tested honey. The three extracts displayed a characteristic profile and vary from each other. This study revealed that bee species is important in determination of honey's quality.

Keywords: *Heterotrigona itama*, *Tetragonula laviceps*, *Geniotrigona thoracica*, stingless bee, DPPH

INTRODUCTION

Stingless bees are part of family Apidae, and closely related to honeybees. Most of bee species were found in tropical countries where approximately 500 species have been recorded worldwide (Heard, 1999; Rasmussen and Cameron, 2007; Schwarz, 1948). It has been reported that more than 30 species were identified in Malaysia. Common species been identified are *Heterotrigona itama, Geniotrigona thoracica, Trigona apicalis, Lisotrigona scintillans, Tetragonula laeviceps, Lepidotrigona terminata, Trigona respani, Trigona melanocephala, Trigona valdezi, Trigona collina, Trigona atripes, Trigona canifrons, Trigona iridepennis* and *Trigona rufibasalia* (Moore, 1961; Mohd Norowi et al., 2008, Rasmussen & Cameron, 2007, Kelly et al., 2014). Stingless bees are important pollinator in tropical rainforest and in agricultural plantations such as star fruit, mango, durian, watermelon, guava and coconut (Eltz et al., 2003; Slaa et al., 2006). The diversity status of stingless bee species depends on several factors such as the variety

of local forest structures and conditions (distance to primary forest, proximity of forest edges and overall habitat diversity) or local disturbance history (Salmah et al., 1990).

Honey is a natural food collected by bees from the nectar of a variety of plants and consists mainly of glucose and fructose. In many cultures, honey mainly used as natural sweetener and also as traditional medicine. Honey is a solution of supersaturated sugar in about 70-80% with nearly 40% of fructose and about 30% glucose (Gheldof et al. 2002). Honey also contains other compounds such as enzymes, amino acids, organic acids, minerals, aromatic substances, vitamins, pigments, beeswax, and pollen. These materials contribute to its color, smell and flavor (Falico et al., 2004; White and Doner, 1980 and Küçük et al., 2007), adding nutritional variety to human diets. The moisture content of honeybee honey is about 17.7%, but for stingless bee honey it can be as high as 30%. The total acidity of honey in general is about 0.08%, and ashes constitute 0.18% (Nagai et al. 2004). Glucose and fructose are the major components of honey and when these crystallize the honey becomes solid, known as granulated honey. There are several factors contributing to the specific composition of honey such as type of vegetation surround the hives, bee species, the climate, environmental and seasonal condition, nectar composition of the plant source and storage condition (Iglesias et al., 2012). Stingless bees' honey is different from that of produced by the bees of the genus Apis (Vit et al., 1994) especially in carbohydrate content and therapeutic effects. The demand for stingless bee honey has increased over the years and has higher prices compared to *Apis melifera* honey. However, compared to *A. melifera* honey, study on stingless bee honey is lacking especially on the physicochemical characteristics of various species. In facts, the current international legislation concerning the beehive products is only directed to the *Apis melifera* products.

In this study, we carried out evaluation on comparison of physicochemical property as well as antioxidant activity of honey produced by three commonly found stingless bees species in Malaysia.

MATERIALS AND METHODS

Collection and Preparation of Honey Extract

Honeys collected by stingless bees *Heterotrigona itama, Tetragonula laeviceps* and *Geniotrigona thoracica* were harvested from University's Apiary, Universiti Sultan Zainal Abidin, Besut Campus, Besut and from YMR Marketing Sdn. Bhd (Dungun, Terengganu). Each sample was kept in 4°C ± 2°C prior analysis. Sample was coded as EHI for *Heterotrigona itama,* ETL for *Tetragonula laeviceps* and EGT for *Geniotrigona thoracica*

Honey samples were extracted following the method described by Vit et al. (1994). Briefly, 50 mL of honeys were diluted with acid water (pH 3.0, adjusted with HCl) until completely fluid. The solutions were then filtered, and the filtrate passed through an Amberlite XAD-2 column (Sigma–Aldrich) prewashed with 100 mL of acid water and subsequently with 300 mL of distilled water. The fractions were then eluted with 300 mL of methanol and then passed through a Sephadex LH-20 column as purifying procedure. These fractions were dried using rotavapor and then stored in the dark at 4°C until used.

Physicochemical Analysis

The physicochemical analysis of the honey samples consisted of the following basic parameters, which was performed in duplicate; ash content, moisture content and total fiber, fat, protein and carbohydrate. The analysis were performed according to method by Almeida-Muradian et al. (2013) with modification.

Determination of Moisture Content

The samples were pulverized if necessary. A crucible was heated in oven at 105°C for 1 hour and then was cooled in desiccator. The cooled empty crucible was weighted until constant weight is achieved and recorded as W1. Accurately weighted of 3g samples were placed and spread evenly across the crucible and weighted as fast

as possible and recorded as W2. The crucibles containing samples were dried at 105˚C per 1 hour in oven. It was then transferred into desiccator. Cooled crucibles were weighted noted as W3. Percentage of moisture was calculated as follows:

Moisture (%) = (W2-W3)/(W2-W1) x 100

Total Protein Content

The nitrogen content was determined using micro Kjeldahl method. One gram of sample was weighted into the digestion tube and two tablets of catalyst Kjeldahl Cu 3.5 were added into digestion tube. Then, twelve milliliters of concentrated H_2SO_4 were added and was gently shake to the wet the samples with the acid and placed into the digestion unit. Twenty five milliliters of receiver solution (twenty five milliliters of boric acid with ten drops of bromocresol green) into the conical flask. The receiver solution in the distillate flask turns to green indicating the presence of alkali-ammonia. The distillate with standardized HCl 0.1N was titrated until the color of mixture turn to red color. Calculation of percentages of protein percentage as follows:

Protein (%) = 0.1 x (mL acid for sample – blank) x 14 x 10 / Weight of the samples x 100

Determination of Fat Content

The extraction cup was dried in the oven for 105˚C for 6 hour a day before experiment and was cooled in desiccator. Pre dried of extraction cup was weighed and was recorded as W2. The sample was weighed (W1) and wrapped with the filter paper and was put into extraction thimble. By using the volumetric cylinder, 110 mL petroleum ether were measured and placed into the extraction cup. The extraction cup was attached to the Automated Soxhlet Fat Extractor. When the extraction was completed, the extraction cup was detached from the extractor. The extraction cup was dried in the oven 105˚C for 2 hour. Next, the extraction cup was cooled in the desiccator and was weighted (W3). Calculation of percentages of fat:

Fat (%) = W3 - W2/ W1 x 100

where,

W1 = weight of the samples
W2 = weight of dry extraction cup
W3 = weight of dry extraction cup + fat

Determination of Ash content

The ash content in the samples was determined by performing the incineration of 1 g of honey (crucible) in a muffle at 550°C for 3 hours. The result is expressed in percentage (%) according to the equation:

Ashes (%) = $[(M_1-M_2)/M_3]$ x 100,

where,

M_1 = weight of the crucible with the ash (g).
M_2 = weight of the crucible (g)
M_3 = weight of honey sample (g)

Determination of Carbohydrate Content

Carbohydrate content was determined by subtracting the amount of ashes, protein and fats from total mass. Total carbohydrate contents was obtained by following equation:

Total carbohydrate (%) = 100 - (ashes + protein + fat) (%)

Determination of Total Phenolic Content

Phenolic compounds from honey samples were estimated by a modified Folin-Ciocalteu method (Silva et al., 2013). Briefly, 100 µL of honey extract were mixed with 200 µL Folin and Ciocalteu's phenol reagent and vortexed to mix well. After 3 min, 800 µL of 10 % Na_2CO_3 solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 2 hours, transfer 200 mL sample, standard or blank from the assay tube to a clear 96-well microplate and read the absorbance of each well at 765 nm by a spectrophotometer (UV-vis mini 1240 Shimadzu Co.). Gallic acid was used to calculate the standard curve (120.0, 160.0, 200.0, 240.0, 280.0 and 320.0 µg/mL). Estimation of the phenolic compounds was carried out in triplicate. The results were reported as the mean ± standard deviations and expressed as micrograms of gallic acid equivalents (GAEs) per mL honey.

Determination of Flavonoids Content

Total flavonoid content (TFC) of EHI, EGT and ETL honey was determined according to the colorimetric assay methods as describe in Ibrahim et al. (2016) and Silva et al. (2013). Honey extract (200 µL) was mixed with 4 mL of distilled water. At baseline, 0.3 mL of $NaNO_2$ (5 %, w/v) was added. After 5 min, 0.3 mL $AICI_3$ (10 % w/v) was added, followed by the addition of 1.5 mL of NaOH (1 M) 6 min later. Immediately after that, the volume was increased to 10 mL by the addition of 2.4 mL distilled water. The mixture was vigorously shaken to ensure adequate mixing, and the absorbance was read at 415 nm. A calibration curve was prepared using a standard solution of Quercetin (12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 µg/mL). The results were also expressed as micrograms of Quercetin equivalents (QEs) per mililitre (mL) honey.

Antioxidant by DPPH Assay

Free radical-scavenging activity of methanolic fractions of the honey samples (EHI, EGT, ETL) was performed according to the procedure of Meda et al. (2005). The methanolic honey elutions were dried with under vacuum using rotary evaporator. The free radical-scavenger activity was determined using the DPPH assay, as described previously (Silva et al., 2006). The antiradical activity was evaluated using a dilution series to obtain five concentrations. This process involved mixing the DPPH solution (60 mM in ethanol) with an appropriate amount of pure honey or extract followed by homogenization. After 30 min, the remaining DPPH radicals were quantified by measuring the absorption at 517 nm using a spectrophotometer. The percentage of inhibition was given by the formula: percent inhibition (%) = $[(A_0-A_1)/A_0]$ x 100, where A_0 was the absorbance of the control solution and A_1 was the absorbance in the presence of the sample and standards. For each sample, assays were performed in triplicate.

Statistical analysis

All the experiments were performed in triplicate (n = 3), and the results were expressed as mean ± standard deviation. Triplicate determinations, mean and standard deviation were calculated. Calibration curve of standard was obtained for concentration versus absorbance. The comparison between different species of stingless bee honey was performed by the One-dimensional Variance Analysis (One-way ANOVA) followed by Tukey test.

RESULTS AND DISCUSSION

Honey is a complex mixture of nectars and plant exudates collected by the bees. It mainly consumed as sweetener as well as for its therapeutic properties. Honey quality parameter was determined according to Codex Alimentarius (CA 2001). However, this quality applicable mainly for honeybee honey but not for stingless bee honeys. There are significant different in various aspect between these two types of honeys. In this study, we analyzed some of the quality parameters onto honeys produced by three different species of stingless bees in order to set the basis of quality criteria for stingless bee honey.

Physicochemical Evaluation

As shown in the Fig. 1, stingless bees honeys have moisture content ranged from 54.66% to 41.49% and was considered high in according to Codex Alimentarius standard. Significant differences were found between the three honeys studied ($p < 0.05$). Honey's moisture content depends on the environmental conditions and the beekeepers' manipulation at the harvest period (Acquarone et al., 2007). Honey having high water content is more likely to ferment, making the preservation and storage more difficult (Iglesias et al., 2012). In addition, our results are in agreement with the studies of Almeida-Muradian et al. (2013), Alves et al. (2008), who have also found higher values of moisture content in stingless bees' honey. However, despite the higher moisture, for reasons that are not yet clear, Melipona honeys are fairly resistant to spoilage by unwanted fermentation (Vit et al., 1994). Figure 1 also shows carbohydrate content ranged from 40.42% to 50.46% of total weight. There were significant differences between the three types stingless bees's honey. *H. itama* have the highest carbohydrate content compared to the other two species. Honey contains a complex mixture of carbohydrates, mainly glucose and fructose that contribute to its color, smell and flavor (Fallico et al., 2004 and Küçük et al., 2007).

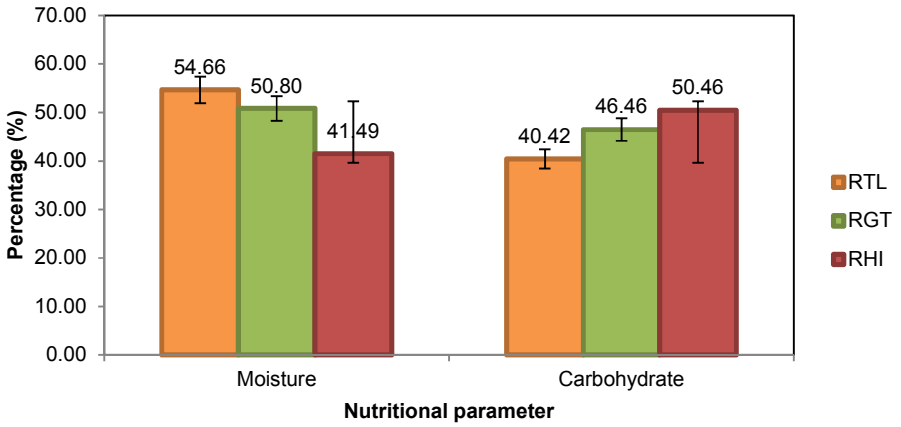

Fig. 1. Moisture content and carbohydrate content for raw honey of RTL, RGT and RHI. RTL- raw honey of *T. laeviceps;* RGT- raw honey of *G. thoracica;* RHI- raw honey of *H. itama*

Based on Fig. 2, the mineral content in honey is generally small and depends on nectar composition of predominant plants in their formation. The soil type in which the original nectar-bearing plant was located also influences the quantity of minerals present in the ash. The ash content of honey is mainly mineral trace elements. Minerals present are calcium, copper, iron, magnesium, manganese, potassium, sodium, and chlorides, phosphates, silicates and sulphates. Dark honeys are often very rich in minerals, but variation in the mineral content of different honeys giving the character to the honey. As such, the variability in ash contents has been associated in a qualitative way with different botanical and geographical origins of honeys (Felsner et al., 2004). As shown in the Fig. 2, different species of stingless bees produced different amount of fat. The fat content of the three species was significant different ($p<0.05$), ranged from 0.46 to 0.96%. Meanwhile, there is no

significant difference of protein content. The value of the protein content in the RTL, RHI and RHI ranged from 0.08% to 0.09% only. It showed that there was very little protein content in the stingless bee's honey. The ash content of RTL, RGT and RGT ranged from 1.39% to 1.96%. However, the value of ash from the previous study was much lesser (0.01%) as reported by Alves et al. (2008), who analyzed stingless bees' honey from different species. The differences in the nutritional analysis of the three types of stingless bees *T. laeviceps*, *G. thoracica* and *H. itama* may be due to the nectar composition of the source plant, bees' species, the climate, environmental and seasonal conditions, agricultural practices and treatment of honey during extraction and storage (Basualdo et al., 2007 and Iglesias et al., 2012).

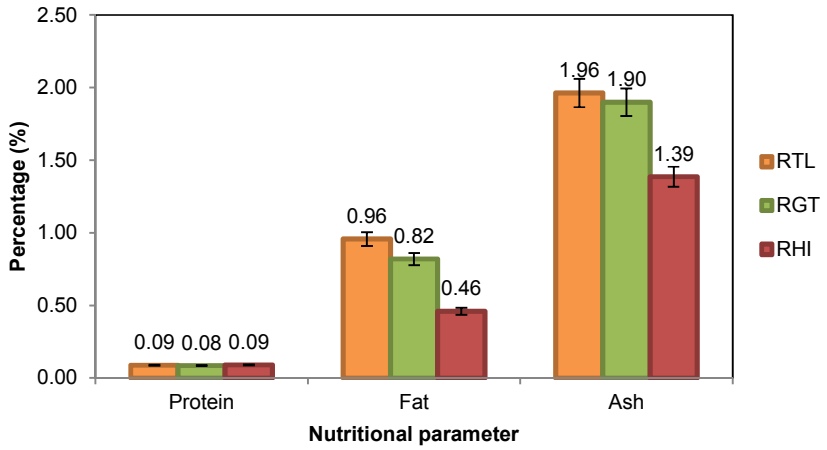

Fig. 2. Protein content, fat content and ash of raw honey of RTL, RGT and RHI. RGT and RHI. RTL- raw honey of *T. laeviceps;* RGT- raw honey of *G. thoracica;* RHI- raw honey of *H. itama.*

Phytochemical Screening

As shown in Fig. 3, the content of total phenolics in the methanol honey extract was determined using the Folin-Ciocalteau reagent. The result of total phenolic content was calculated from the regression equation of the standard plot ($y=0.01514x+0.07169$, $r^2=0.994$) and is expressed as Gallic acid equivalent (GAE) (Fig. 3a). It is shown that the highest concentration of phenolic possess by *T. laeviceps* (ETL) honey. Phenolic compounds in plants play the key role as primary antioxidants or free radical scavengers as well as other biological activities. The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase and scavenge free radical (Mallavadhani et al., 2006). TPC is considered as a fast and simple method to measure the total phenol in complex matrix like honey. TPC method was sensitive enough for total phenol estimation in honey samples (Al et al., 2009).

Flavonoid content was calculated from the regression equation of the standard curve, $y = 0.033x+0.044$, $r^2=0.993$) and expressed as Quercetin equivalents (QE) (Fig. 4a). Flavonoids are the most common and widely distributed group of plant's phenolic compounds, characterized by a benzo-γ-pyrone structure (Manik et al., 2013). The TFC of honey samples was determined based on the method of aluminium chloride ($AlCl_3$), which was specific for flavones and flavonols (Alvarez-Suarez et al., 2010). Based on Fig. 4b, honey extracts had significant differences from each other but there no significant in total flavonoid content of different raw honey. *T. laeviceps* showed the highest concentration of flavonoid content which are 5.04% compared to others.

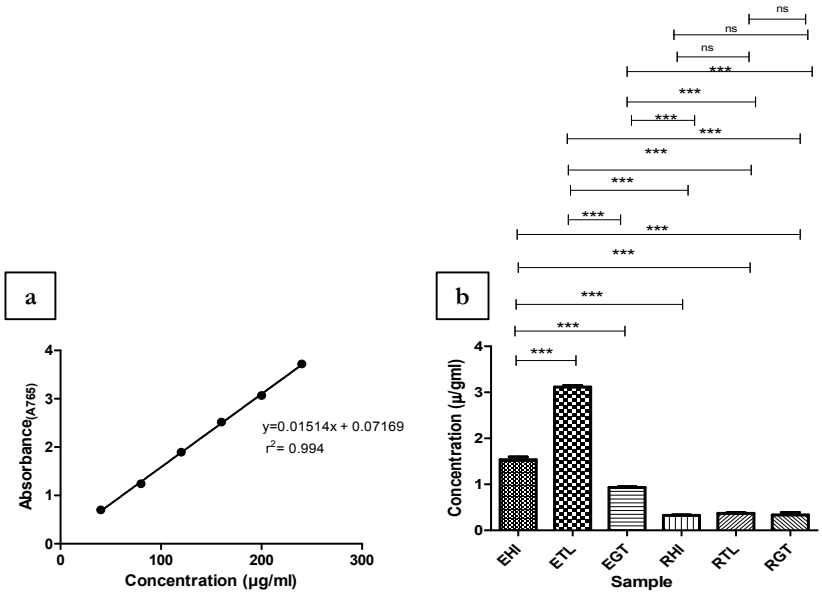

Fig. 3. Standard curves of a) Gallic acid. Graph column of b) Total phenolic Content with Gallic Acid Equivalent. The graph of TPC showed that there were significant different between all samples. The [***] indicate the samples are highly significant different which p < 0.0001. RTL- raw honey of *T. laeviceps;* RGT- raw honey of *G. thoracica;* RHI- raw honey of *H. itama.* EHI- *H. itama* honey extract; ETL- *T. laeviceps* honey extract; EGT- *G. thoracica* honey extract.

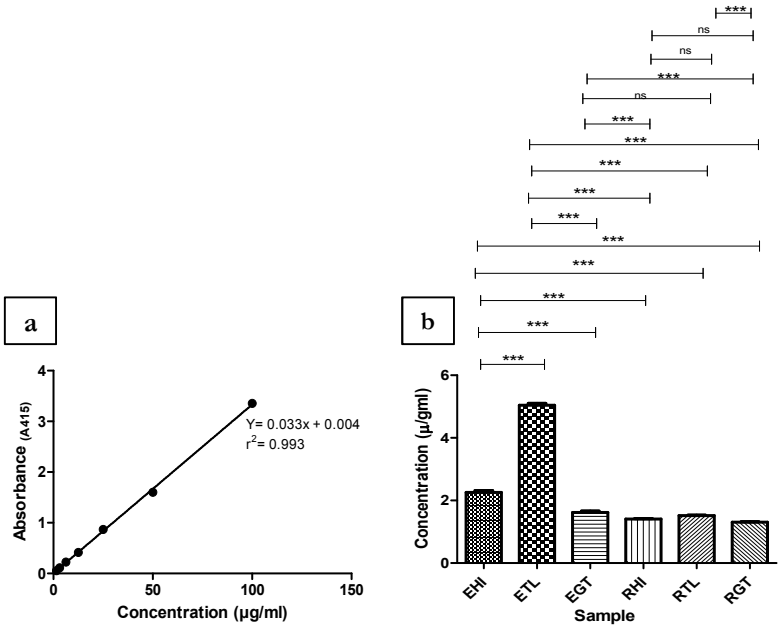

Fig. 4. Standard curves of a) Quercetin. Graph column of b) Total Flavonoid Content with Gallic Acid Equivalent. The graph of TFC showed that there were significant different between all samples. The [***] indicate the samples are highly significant different which p < 0.0001. RTL- raw honey of *T. laeviceps;* RGT- raw honey of *G. thoracica;* RHI- raw honey of *H. itama.* EHI- *H. itama* honey extract; ETL- *T. laeviceps* honey extract; EGT- *G. thoracica* honey extract.

The different may due to the different type of stingless bees species and their botanical origin. (Basualdo et al., 2007; Iglesias et al., 2012). Several studies reported that flavonoids present in herbs significantly contributed to their antioxidant properties (da Silva et al., 2013; Ferreira et al., 2009). It has been shown to be highly effective scavengers of most oxidizing molecules, including single oxygen and various free radicals.

Antioxidant activity

The scavenging activity of honey samples had been measured by using DPPH assay, and trolox was used as positive control. The unpaired electron of DPPH forms a pair with a hydrogen donated by free radical scavenging antioxidant from honey and thus converting purple coloured odd electron DPPH to its reduced form in yellow. The degree of decolourization was measured spectrophotometrically in order to determine the scavenging activity of honey. Fig. 5 indicates that ETL honey has the highest scavenging activity, followed by EHI and EGT honey samples.

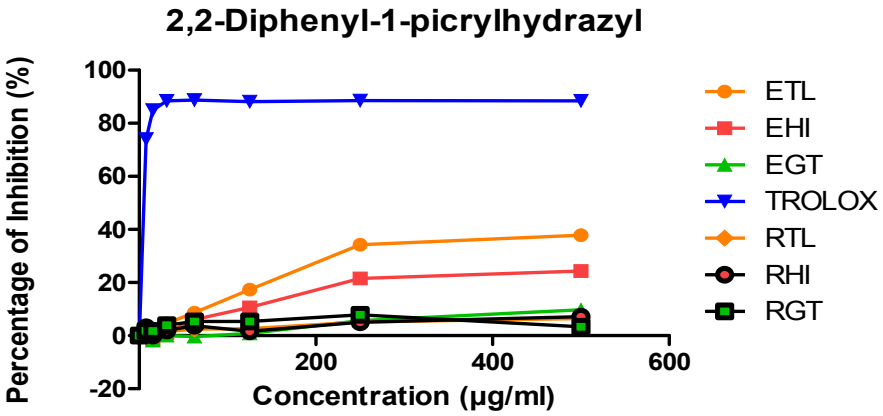

Fig. 5. DPPH scavenging activity by stingless bee honey of ETL, EHI, EGT, RTL, RHI, RGT and Trolox. RTL- raw honey of *T. laeviceps;* RGT- raw honey of *G. thoracica;* RHI- raw honey of *H. itama.* EHI- *H. itama* honey extract; ETL- *T. laeviceps* honey extract; EGT- *G. thoracica* honey extract. Trolox served as standard.

Thin layer Chromatography (TLC) Analysis

TLC methods were performed for the separation of compound in the EHI, ETL and EGT extracts. The mobile phase used was Hexane: ethyl acetate: formic acid with ratio 4: 6: 0.1 (v/v). Based on the Fig. 6, it was shown that the compound was visible under short wave and long wave of UV light. The plate also visualized after derivatized by anisaldehyde reagent (Fig. 6c). There are 2 major spots occur in EHI, at R_f of 0.7 and 0.8 visualized under UV 254 nm. Whilst, only one major spot occur in ETL at of R_f 0.70, visualized under the same wavelength and there is no major spot occur in EGT. After derivatization, revealed complex mixture of compounds, which exhibit different colored reactions. There were some differences as well as similarities between to samples. From the Fig. 6c, EHI revealed a dark purple violet with R_f value at 0.7, which were also observed in ETL. Also in Fig. 6c, both samples turn purple upon derivatisation. Overall, from TLC analysis, the chemical compositions of honey produced by *H. itama* are more complex compare to honey produce by *T. laeviceps* and *G. thoracica.*

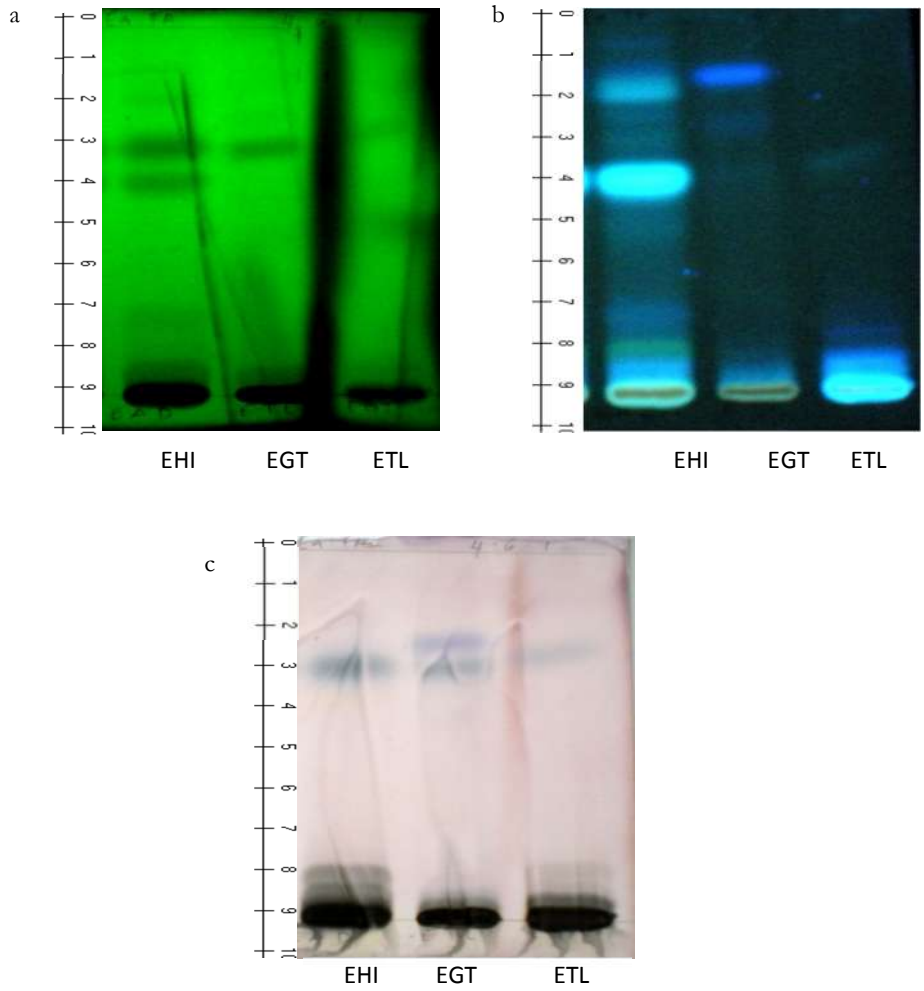

Fig. 6. TLC photodocumentation of methanolic extract EHI, EGT and ETL honey by visualization (a) UV 254 nm (b) UV 366 nm (c) Post derivatisation with anisaldehyde reagent.

CONCLUSION

Finding from this study revealed that bee species is important in determination of honey's quality. Nutritional content analysis shows there were differences between species. *Tetratrigona laeviceps* possess the highest value in total phenolic content and total flavonoid content compare to others. However, honey extracts from all species showed low antioxidant activity. Chemical composition of honey extracts also different according to species with *H. itama* showed the most number of compounds based on TLC analysis. More analysis need to be carried out in order to further established the differences of honey produced by different bee species.

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