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Physicochemical Analysis, Antioxidant and Anti Proliferation Activities of Honey, Propolis and Beebread Harvested from Stingless Bee

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Abstract Stingless bees (Kelulut) can produce three major commercial products i.e. honey, propolis and beebread. These products are widely believed to have medicinal benefits, similar with products produced by stinging bees. However, there are very few scientific data available on the stingless bee's products to prove the claims. Thus, this study was conducted to investigate the characteristics of the products from the perspective of physicochemical analysis, activities of antioxidant and anti proliferation on cancer cells. Stingless bees honey, propolis and beebread were collected and their physicochemical and antioxidant properties were analysed prior to treatment on human breast adenocarcinoma (MCF-7) cell lines. Physicochemical analysis indicated that the samples are mostly not within the range reported by the Codex Standard for Honey. Honey, propolis and beebread exhibited antioxidant activity through the total phenolic content of 700 mg GAE/kg, 1600 mg GAE/kg and 300 mg GAE/kg respectively. Propolis has the highest antioxidant activity and inhibited MCF-7 cell growth at IC_{50} of 38.9 µg/ml. Meanwhile, stingless bee honey and beebread displayed the IC_{50} at 60 v/v and 64µg/mL respectively. The data is crucial to unveiled and prove medicinal properties and potential possessed by the stingless bee products. Subsequently, increase their commercial value in the future.

1. Introduction

Stingless bee or locally known as Kelulut is one of the native bee species available in Malaysia (Ismail, 2016). The bee can serve as a pollination agent and intriguingly it also can produce three major products, which are honey, propolis and beebread. The products, in particularly honey have high economic potential because of aesthetic taste (sweet and sour) compared to the conventional honey (sweet). In addition, many claims have been made related to the medicinal values. However, the data is still limited and is yet to be discovered at present.

Honey is classified as a functional food because of its outstanding chemical compositions including antioxidants (Bogdanov et al., 1999). Meanwhile, propolis is made up of plant resins and beeswax, which are masticated by the stingless bee using its salivary enzymes to build the hive pots. Beebread is a fermented mixture of plant pollen, honey, and saliva of bees, which is used as a major protein source to the bees. Various chemical compositions found in honey, propolis and beebread made the stingless bee's products unique with specific characterisations. The characterisation can be evaluated using physicochemical analysis such as moisture content, ash content, pH, free acidity, hydroxymethylfurfural (HMF), wax content, level of protein and carbohydrate. Method for these analyses are acknowledged globally to observe the quality of product and even used to differentiate between natural and adulterated honey (Bogdanov, 2009; Samat et al., 2018).

Many consumers believe in the medicinal value of stingless bee products. However, data on the medical properties of stingless bee products is still limited. In order to prove the claims, the antioxidant properties of all the stingless bee products in the study were tested using total phenolic content and on their anti-proliferation of cancer cell lines.

2. Materials and Methods

2.1. Sample collection

Honey and propolis were collected from species of stingless bee, *Geniotrigona thoracica*. Meanwhile, beebread was harvested from stingless bee *Heterotrigona itama* from Humaira' Honey kelulut farm in Lenggong, Perak. The samples were stored in a refrigerator at 4°C prior to use.

2.2. Sample preparation

In this study, honey sample can be used readily for the experiment. However, extraction procedures were applied on propolis and beebread prior to testing on physicochemical and antioxidant analyses, and on cancer cell lines. 50 g of ground propolis was mixed with 500 ml of 80% ethanol. Then, the mixtures were shaken at 200 rpm for 48 h at 37°C. Next, it was spn at 3000 rpm for 15 min at 10°C. Then, the filtrate was evaporated using a rotary evaporator at 175 mbar and at a temperature of 52°C at 95 rpm (Jacob et al., 2015). The propolis ethanolic extract was kept in a chiller at 4°C for further use. For the extraction process of beebread, the sample was dried before it can be crushed. Next, 20 g of sample were mixed with 80 mL of 95% ethanol and then put on a shaker at 200 rpm and 37 °C for 24 h. The top layers were decanted and centrifuged at 3000 rpm for 30 min at 20°C. After that, the supernatant was evaporated at 40°C in the rotary evaporator. The pressure and rotation were set at 156 mbar and 950 rpm respectively. The end product was weighed and stored in the dark at 8°C (Markiewicz-Zukowska et al., 2013).

2.3. Physicochemical analyses

Physicochemical analyses were conducted according to the methods defined in the IHC (Bogdanov, 2009), Samat et al., (2014) and Bogdanov et al., (1999). These include moisture, ash content, pH, free acidity and hydroxymethylfurfural (HMF) level for honey. Meanwhile for propolis, physicochemical analyses were conducted on the content of moisture, ash and wax. Physicochemical analyses of beebread were carried out on moisture and ash content; and the level of protein and carbohydrate found in the sample.

2.3.1 Moisture content

The moisture content of honey was measured using a portable digital refractometer (MISCO refractometer, United States) at 20^{o}C. For propolis, 10g of ground propolis was heated to 100°C for 5 h in the oven. Then, the heated sample was desiccated until constant weight to measure the final weight of the sample (Cunha et al., 2004). For beebread, 1 g of sample was put in a hot-air oven at 90°C for 4 h and 48 min. Then, the samples were removed from the oven and they were allowed to reach room temperature and then weighed. The difference between dry weight and initial weight was used to calculate moisture content (Carpes et al., 2009).

2.3.2 Ash content

Ash content of honey was determined readily using electrical furnace at temperature (450-550^{o}C) for 15 min (Bogdanov, 1999). 2g ground propolis and beebread were burnt separately at a temperature of 600°C for 1 h using the furnace (Carpes et al., 2009, Cunha et al., 2004).

2.3.3 pH and free acidity

10 g of honey was dissolved in 75 ml of distilled water before the pH was measured using a Delta 320 pH meter (Mettler Toledo, Switzerland). Then, the dissolved honey solution was titrated with 0.1 M sodium hydroxide (NaOH) until pH 8.3 to obtain the free acidity value of honey using the same device.

2.3.4 Hydroxymethylfurfural (HMF)
HMF was determined as per method outlined by White (1979). A total of 5 g honey was diluted with 25 ml distilled water and transferred prior to adding 0.5 ml of Carrez I and Carrez II solution. Distilled water was then added up to 50 ml before it was filtered using a 0.45 µm filter paper. Discard the first 10 ml filtrate. 5 ml honey solution was mixed with 5 ml water before measuring its absorbance at 284 nm and 336 nm against reference solution of 5 ml initial honey solution and 5 ml 0.2% sodium bisulphite solution. The values were then expressed in mg/kg.

2.3.5 Wax analysis
Propolis extract was left in the freezer overnight before it was filtered and its wax composition weighed based on the method described by Cunha et al., (2004).

2.3.6 Protein content
Bradford protein assay was used to determine the protein content. 100 µL of beebread sample was added to 9.9 mL distilled water. Next, 3 drops of Bradford's reagent was added. Then, the absorbance was measured at 595 nm after 10 min. Protein standard curve using bovine serum albumin (BSA) was used to determine the content of protein in the beebread sample. The protein standard curve was prepared using a protein stock solution (0.01 g/mL). The final concentrations of protein (g/mL) that were used are 0, 0.00125, 0.0025, 0.005 and 0.01 (Bradford, 1976).

2.3.7 Carbohydrate content
Somogyi-Nelson method was used to determine carbohydrate content. 100 µL of beebread sample was added to 9.9 mL of distilled water. Next, 1 mL of Nelson reagent was added. Afterwards, the solution was placed in boiling water for 20 minutes and then cooled to room temperature. Next, 1 mL arsenomolybdate reagent solution was added. The absorbance was measured at 510 nm using a spectrophotometer. Carbohydrate standard curve using glucose was used to determine the content of carbohydrate in beebread sample. The standard curve was prepared using a carbohydrate stock solution (10 mg/mL). The final concentrations of carbohydrate (mg/mL) that were used are 0, 1.25, 2.5, 5 and 10 (Carpes et al., 2009).

2.4 Antioxidant level
Total phenolic content of honey, propolis and beebread sample were estimated by using a modified spectrophotometric Folin-Ciocalteu method. Gallic acid was used as a standard by using several concentrations (1.25 mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml). 2 g of honey and 200 µl of propolis and beebread respectively from stock solution was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. 1 ml of 10% sodium carbonate solution was added to the mixture after 3 min. The volume of the mixture was adjusted to 10 ml by using distilled water. The reaction was left in the dark for 90 min. The absorbance of the sample was read at 725 nm (Ibrahim et al., 2015).

2.5 Anti proliferative Study
MCF-7 cell lines were purchased from ATCC (USA). The cells were treated with 0, 25, 50, 75 and 100 v/v of honey and 0, 25, 50, 75 and 100 µg/ml of propolis and beebread respectively for 72 h. Untreated cells were used as a negative control. Meanwhile, hydrogen peroxide (H_2O_2) was selected as a positive control. Anti proliferation activity was measured using MTT assay. The absorbance was read at a wavelength of 570 nm by using a microplate reader (Mohd Salleh et al., 2017).

2.6 Statistical analysis
The values were expressed as the mean ± standard error of the mean (SEM). Then, IC_{50} value from anti proliferation study was calculated using GraphPad Prism 5.0. One-way ANOVA with Tukey HSD test were used to analyse the anti proliferative results. A level of probability of $p < 0.05$ was set as statistically significant (Mohd Radzman et al., 2013).

3. Results

3.1 Physicochemical analyses and antioxidant level

Physicochemical analyses were conducted to characterize honey and propolis samples harvested from *G. thoracica* beehive, and beebread collected from *H. itama* (Table 1). The data showed that honey contained the highest level of moisture, followed by beebread and propolis. Propolis has the highest ash content while honey exhibited the lowest ash content. In terms of total phenolic content, propolis showed the highest antioxidant properties, followed by honey and beebread.

Table 1. Physicochemical analyses and antioxidant level of honey, propolis and beebread samples harvested from stingless bee

Sample	Moi-sture (%)	Ash (g/100g)	pH	Free acidity (meq/ kg)	HMF (mg/ kg)	Wax (%)	Protein (%)	Carbo-hydrate (%)	TPC (mg GAE/kg)
Honey	28.78 ± 3.8	0.22 ± 0.0	3.21 ± 0.0	58 ± 0.5	47 ± 1.1	nd	nd	nd	700 ± 0.0
Propolis	6.42 ± 0.0	3.85 ± 0.0	nd	nd	nd	33.7 ± 0.7	nd	nd	1600 ± 0.0
Beebread	8.10 ± 0.1	1.70 ± 0.0	nd	nd	nd	nd	47.4 ± 0.0	55.1 ± 2.4	300 ± 0.0

nd = not determined

3.2 Anti proliferation activity

Stingless bee honey demonstrated anti proliferative activity on MCF-7 cell lines at IC_{50} = 60v/v (Figure 1). Meanwhile, propolis and beebread showed the IC_{50} = 38.9µg/ml (Figure 2) and 64µg/ml (Figure 3) respectively. Based on the results, honey, propolis and bee bread showed dose dependent effects and significantly inhibited the cell cancer growth.

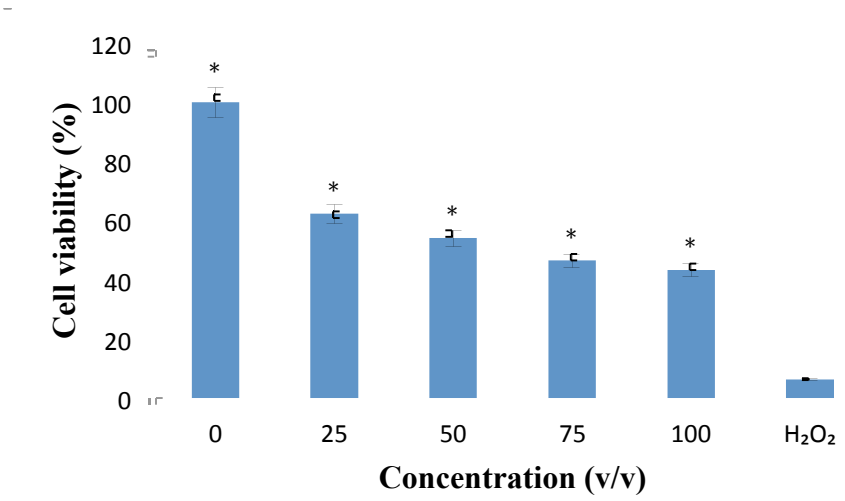

Figure 1. Anti proliferative activity of stingless bee honey from *G. thoracica* on MCF-7 cell lines after being treated with several concentrations of honey (0-100%) for 72 h. The analysis was performed using one-way ANOVA analysis (Tukey HSD test). * indicates $p < 0.05$ significant difference of the concentration towards the control. n = 3.

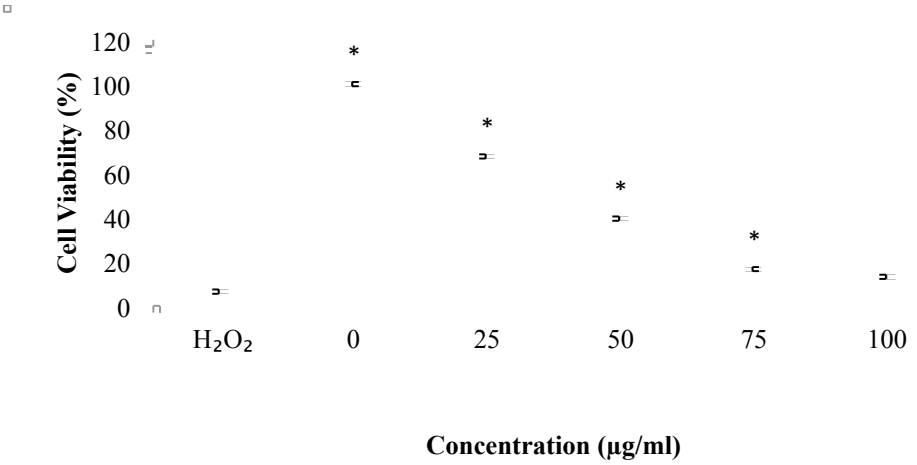

Figure 2. Anti proliferative activity of stingless bee propolis from *G. thoracica* on MCF-7 cell lines after being treated with several concentrations of propolis (0-100%) for 72 h. The analysis was performed using one-way ANOVA analysis (Tukey HSD test). * indicates $p < 0.05$ significant difference of the concentration towards the control. n = 3.

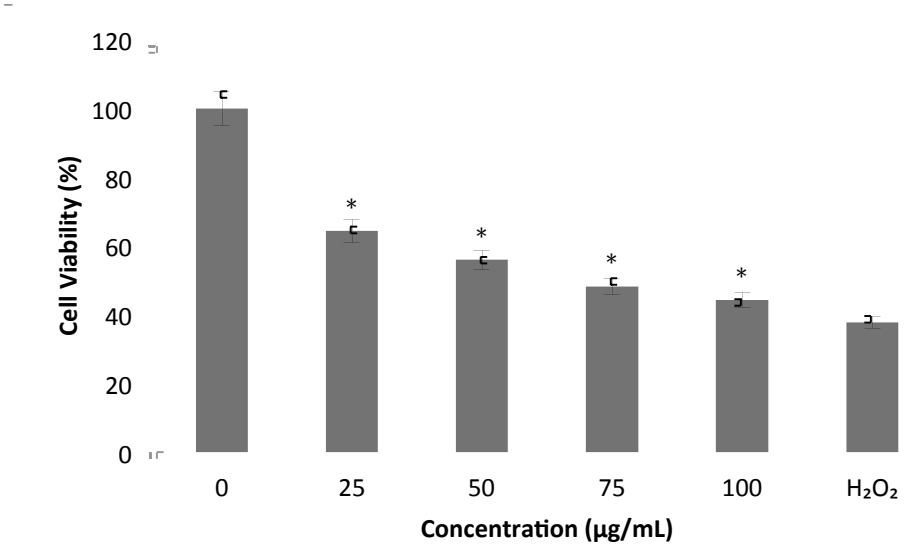

Figure 3. Anti proliferative activity of stingless bee beebread from *H. itama* on MCF-7 cell lines after being treated with several concentrations of bee bread (0-100%) for 72 h. The analysis was performed using One-way ANOVA analysis (Tukey HSD test). * indicates $p < 0.05$ significant difference of the concentration towards the control. n = 3.

4. Discussion

Most of the data obtained from physicochemical analyses on honey, propolis and beebread of stingless bee are not within the range of values set by the Codex Standard for Honey (Codex Alimentarius Commission, 1981) and other studies (Kieliszek et al., 2018 and Stan et al., 2011). These findings as expected because data set in the codex are based on honey from bee species of *Apis* (stinging bee) particularly from temperate countries. Meanwhile, our samples are collected from other bee species from tribe Meliponinae, which are a stingless bee. In addition, the stingless bee is only available in the tropical countries including Malaysia. The finding has revealed that stingless bee products have their

own physicochemical characteristic and the differences need to be recognized worldwide for commercial purposes.

Each sample were subjected to different physicochemical analyses (some data were not determined-nd) as some analyses are more relevant towards certain samples, for example wax content analysis on propolis and protein and carbohydrate content on bee bread (Campos et al., 2008). The wax content of propolis used in this study is considered high (33.7%) since it is close to the 38% limit (Stan et al., 2011) and higher than the Brazilian propolis samples (Maria et al., 2016). Protein and carbohydrate content of the beebread sample are also higher compared to the 25.9% protein and 48.8% carbohydrate reported for beebread from stinging bee (Carpes et al., 2009).

Propolis has the highest antioxidant level, followed by honey and beebread. This is in agreement with another study by Bakchiche et al., 2017, which reported that propolis has higher TPC compared to honey. All samples had higher antioxidant content compared to reports from similar bee products produced by the stinging bee species *Apis mellifera* (Silva et al., 2013; Sime et al., 2015). This might be due to the differences in pollen foraging activities of singing and stingless bee products (Nurdianah et al., 2016), weather and geographical location (Markiewicz-Zukowska et al., 2013) and also differences in the solvent used for extraction (Wan et al., 2014).

Amongst the bee products, propolis with the highest antioxidant level exhibited the lowest IC_{50} on the proliferation of MCF-7 cell lines. This might be the existence of specific phenolics or flavonoids, or synergism between several compounds that are responsible for the low inhibitory concentration (IC_{50}) on MCF-7 cell lines in the propolis. However, the findings are required further investigation and validation.

5. Conclusion

Stingless bee products have unique physicochemical characters. Sample with the highest antioxidant level, propolis (1600 mg GAE/kg) has been shown to exert the lowest IC_{50} value (38.9µg/ml) on MCF-7 cell lines. Further analysis on the properties and biological activities of stingless bee products are crucial to highlight their potential benefits and at the same time promoting and increasing their commercial value in the future.

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