Antioxidant, Antibacterial and Anti-Diabetic Activities of Stingless Bee Honey from Selected Areas in Peninsular Malaysia

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Abstract. Stingless bee honey is one of the Malaysian honeys that has many benefits especially medically, due to the presence of enormous active phytochemical compounds. The active compounds in the honey vary depending on its geographical origin, especially the environmental conditions of the plant nectar. This study aims to analyze and compare the antioxidant, antibacterial and antidiabetic activities of stingless bee honey from different areas in Malaysia. The sample stingless bees’ honeys were obtained from Kulim, Kedah (honey sample 1), Tanjung Malim, Perak (honey sample 2) and Kuala Selangor, Selangor (honey sample 3). The methods used for the analysis were DPPH free radical scavenging, Kirby-Bauer disc diffusion assay and alpha-amylase inhibition assay for the respective activities. An analysis of gallic acid content of the stingless bee’s honeys were also performed using high-performance-liquid-chromatography (HPLC). Tukey’s multiple comparison test was used for statistical analysis. The results indicated that honey sample 2 exhibited the highest antioxidant activity with IC50 values of 89.04 ± 0.83 mg/mL, whilst honey sample 3 showed the highest inhibition capability on both E. coli and S. aureus at 16.33 ± 3.06 mm and 22.67 ± 0.58 mm respectively as compared to the other honey samples. Honey sample 3 also showed the highest inhibition against alpha amylase with an IC50 value of at 15.80 µg/mL. The gallic acid content of honey sample 3 also the highest at 39.79 µg/mL. As a conclusion, honey sample 3 originated from Kuala Selangor, Selangor has better antibacterial and anti-alpha amylase activities. Meanwhile, honey sample 2 originated from Tanjung Malim, Perak has better antioxidant properties.

1. Introduction
Malaysia is one of the honey production countries due to suitable living conditions for bees. The rainforest tropical climate of Malaysia allows the honeybees to have optimal warm temperature and high humidity throughout the year with an average temperature at 27°C and rainfall at 250 centimetres [1]. Stingless bee honey is produced from the nectar of flowers or another part of the tree by a stingless bee (Trigona spp.). There are approximately 17-32 Trigona species are commonly used to produce stingless bee honey in Malaysia. The honey is mainly made up from carbohydrates which are monosaccharides, hexoses, fructose, glucose, sucrose and water. It is also rich in minerals, proteins, free amino acids, enzymes and phytochemicals such as phenolic compound and flavonoids [2]. The physical and chemical properties of honey harvested are varied due to the environmental conditions of the plants [3]. Stingless bee honey has distinctive colour, taste and viscosity compared to other types of honey such as Tualang, Manuka and Gelam honey. Stingless bee honey is also believed to have
high moisture content and acidity due to the presence of organic acids and minerals [4].

Honey has been used as remedies centuries ago and is still being included in both traditional and modern medical practices. The biological properties of honey may include activities such as antimicrobial, anticancer, anti-inflammatory, anti-diabetic, wound healing properties [5], [6]. Most of the biological properties of honeys are attributed to the presence of polyphenols, such as flavonoids. However, the combination of compounds presents in honey such as phenolic acids, flavonoids, ascorbic acid, catalase and peroxidase and seems to more significantly contribute to the antioxidant activity in honey [7]. Meanwhile, the antibacterial activity in honey is attributed by high osmolarity and high acidity of honey, as well as the presence of bee defensin-1 and antibacterial peptides [8]. Another therapeutic property of honey is anti-diabetic, in which honey could lower the glycemic index in type I diabetic patients and did not cause acute hyperglycemic effects on type II diabetics [9].

Although it is well-known that honey has many beneficial effects due to the presence of phenolic compounds, the activities will vary due to the floral source, honeybee, geographical area and climate of the origin of honey [10]. In this study, stingless bee honey from different areas in Peninsular Malaysia was investigated and compared for their antioxidant, antibacterial and anti-diabetic activities. Stingless bee honey was selected due to higher therapeutic values compared to other honey types [11], [12]. Furthermore, very limited studies are presence on geographically different source of stingless honeybee in Malaysia. Thus, this present study will benefit the honey farming industry, especially when selecting area for stingless bee farm in order to produce high quality honey.

2. Materials and Methods

2.1. Chemicals and Reagents
Methanol (purity: ≥99.9%), ethanol (purity: 99.2%), 2, 2-diphenyl-1-picrylhydrazyl-hydrate (DPPH) powder, ascorbic acid, gallic acid, starch, maltose, dinitrosalicylic acid colour reagent (DNS), chloramphenicol, sodium hydrogen phosphate (Na2HPO4), disodium phosphate (Na2PO4), sodium chloride (NaCl), sodium phosphate tartrate tetrahydrate were purchased from Sigma-Aldrich, Germany. Nutrient agar and nutrient broth were purchased from HiMedia Laboratories, India. Porcine pancreatic α-amylase was purchased from Kyron Labs, South Africa.

2.2. Collection of Samples
Stingless bee honey samples were collected from three different areas in Peninsular Malaysia, which were Kulim, Kedah (honey sample 1), Tanjung Malim, Perak (honey sample 2) and Kuala Selangor, Selangor (honey sample 3). The honey samples were stored in closed glass bottle and kept at room temperature to avoid excessive heat. The location of Kulim, Kedah is at latitude 5.3981576 and longitude 100.5705552. Tanjung Malim, Perak is located at latitude 3.7399519 and longitude 101.5338731. Kuala Selangor, Selangor is located at latitude 4.1490556 and longitude 100.1321918.

2.3. Extraction of Samples
Stingless bee honey samples were prepared according to the previous methods [13]. Sample (10 g) was diluted with methanol (25 mL) in a falcon tube. The mixture was mixed using a vortex and centrifuged (3000 rpm) at 25 °C for 10 min. The supernatant was collected in a 50 mL beaker and was dried in the oven at 40 °C for 5 h. The crude honey extract was kept in the refrigerator at 4 °C until use. All steps were repeated for the other honey samples.

2.4. Determination of Antioxidant Activity
The previous methods were followed with minor modification for antioxidant assay [14]. Briefly, ascorbic acid standards were prepared at 0.625, 1.25, 2.5, 5, 10, 15 and 20 μg/mL in methanol. Honey extract were prepared at 10, 20, 40, 80 and 160 mg/mL in methanol. 2 mL of DPPH solution in methanol (20 mg/L) was transferred into 1 mL of methanolic honey solution at different concentrations. The reaction mixtures were shaken and kept for 30 min in the dark at room
temperature. The absorbance (OD) of mixtures was measured using UV-Visible spectrophotometer at 517 nm. The experiment was performed in triplicate. The steps were repeated for ascorbic acid as a positive control. The scavenging activities of the honey extract and ascorbic acid were calculated as below:

\[
\text{Percentage DPPH scavenging (\%) = } \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100\%
\]

2.5. Determination of Antibacterial Activity
The previous methods were modified for antibacterial assay [15], [16]. Sample was prepared at two concentrations (1000 and 500 mg/mL). 28 g of agar powder was dissolved in 1000 mL distilled water, then autoclaved at 80 °C for an hour. Staphylococcus aureus and Escherichia coli were prepared from stock culture by streaking a loop of each bacteria onto the agar. Bacteria culture with the same morphology was selected for the test. The disc (6 mm) was soaked with 20 μL of honey samples, allowed to dry and carefully placed on the cultured agar plate. The agar plate was incubated at 37 °C for 24 h. Chloramphenicol (17 mg/mL) and sterilized distilled water was used as a positive and negative controls. The experiment was performed in triplicates. The size of the inhibition zone was measured in millimetre (mm).

2.6. Determination of Anti-diabetic Activity
The previous methods for alpha-amylose inhibition assay was followed with modification [17]. 0.02 M (pH 6.9) was prepared and the pH was adjusted using Na₂HPO₄ and Na₂PO₄ [18]. Porcine pancreatic α-amylase (0.5 mg/mL) and starch solution (0.01 g/mL), as substrate were prepared using the sodium phosphate buffer. Samples was prepared at 2.5, 5.10 and 20 μg/mL in methanol and was pipetted (500 μL) into test tubes followed by the alpha-amylose solution (500 μL). The mixture was pre-incubated at 25 °C for 10 min. After incubation, 500 μL of 1% starch solution was added into the test tubes and further incubated at 25 °C for 10 min. The reaction was stopped with 1.0 mL of DNS acid colour reagent. The incubation process was continued in the boiling water bath for 5 min before cooled at room temperature and diluted with 10 mL of distilled water. The absorbance (OD) of the mixtures was measured using UV-Visible spectrophotometer at 540 nm. The maltose and methanol were used as background control. The alpha-amylose inhibition percentage was calculated as below:

\[
\text{Percentage enzyme inhibition (\%) = } \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100\% \quad \text{(2.2)}
\]

2.7. Determination of Phytochemical
Previous methods as in section 2.3 was followed with modification. The dried honey samples were diluted in methanol at 1000, 500 and 250 mg/mL and were filtered through a 0.45 mm Millipore filter (Millipore Corp., Bedford, MA). High-Performance Liquid Chromatography (HPLC) analysis of stingless bee honey was performed on an LC-10A series liquid chromatography (Shimadzu, Japan) using detector is Diode Array Detector (DAD) and C₁₈ column [19]. The gradient elution during the processing was 20% for solvent A (3% acetic acid in deionized water) and 80% solvent B (80% methanol in deionized water). HPLC was set at 35 °C, 0.8 mL/min flow rate, 38 °C column temperature, 5 μL injection volume, 7 min time of processing and was monitored at 245 nm. The presence of gallic acid in samples were obtained by comparing the retention times of gallic acid standard.

2.8. Determination of Air Pollution Index
The air pollution index (API) was determined from Air Program Information Management System [20]. API was observed a week before the date of harvested to see the correlation between air condition of the honey’s geographical origin with biological activities and gallic acid content in stingless bee honey. The air pollutant index scale and description of the air quality levels are as in Table 1.
Table 1. The air pollution index scale

| API (µg/m³) | 0 – 50 | 51 – 100 | 101 – 200 | 201 – 300 | >301 |
|------------|--------|----------|-----------|----------|------|
| Status     | Good   | Moderate | Unhealthy | Very Unhealthy | Hazardous |

Source: Department of Environmental (2019)

2.9. Statistical Analysis
Analysis of variance (ANOVA) was performed in Graphpad Prism 7 and \( p \leq 0.05 \) was considered as significant.

3. Results and Discussion

3.1. Antioxidant Activity
IC\(_{50}\) value was calculated for each honey samples and standard, and the results were tabulated in Table 2. The IC\(_{50}\) values were the concentration that caused 50% scavenging of free radicals. The lower the IC\(_{50}\) value, the higher the antioxidant activity. All stingless bee honey samples showed antioxidant activity with the highest activity exhibited by honey sample 2 > honey sample 3 > honey sample 1. However, the antioxidant activity of all stingless bee honey samples is lower than ascorbic acid. All the honey samples and standard showed high scavenging capacity with increases concentration and the data was coherent with previous studies [14], [17], [21].

Table 2. The IC\(_{50}\) of DPPH Scavenging % of different samples. Different alphabets indicate significant difference and similar alphabets indicate no significant difference at \( p<0.05 \), where Tukey test with multiple comparisons were performed.

| Sample                  | IC\(_{50}\) Value ± SD (mg/mL) |
|-------------------------|--------------------------------|
| Honey Sample 1          | 106.72 ± 7.57 \(^a\)           |
| Honey Sample 2          | 89.04 ± 0.83 \(^b, d\)         |
| Honey Sample 3          | 96.59 ± 2.66 \(^a, d\)         |
| Ascorbic acid           | 0.02 ± 0.01 \(^c\)             |

3.2. Antibacterial Activity
Table 3. shows the zone of inhibition (mm) of three stingless bee honey sample against \( S. \) aureus (Gram positive) and \( E. \) coli (Gram negative) in triplicates. The data shows a concentration-dependency activity. Higher concentration exhibited larger zone of inhibition except for honey sample 1. Inhibition against \( E. \) coli was honey sample 3 > honey sample 2 > honey sample 1 at 1000 mg/mL. For the \( S. \) aureus, both honey sample 2 and 3 showed larger inhibitions than honey sample 1 at 1000 mg/mL. Overall, stingless bee honey sample 3 had the highest capability for inhibiting both \( E. \) coli and \( S. \) aureus with higher activity against \( S. \) aureus compared to the \( E. \) coli. The antibacterial activity of honey has been shown to be attributed by hydrogen peroxide [8], as well as phenolic acid such as gallic, caffeic, benzoic, ferulic and cinnamic acid [22]. Similarly, an analysis of correlation between gallic acid content (Table 3.4) and antibacterial activities in this study also showed the same positive correlation, where honey sample 3 has the highest gallic acid.
Table 3. Mean ± SD (mm) of zone inhibition of honey samples at two different concentrations; 1000 and 500 mg/mL against two bacteria; Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus).

| Sample       | Bacteria | Honey Concentration (mg/mL) | Negative Control | Positive Control |
|--------------|----------|-----------------------------|------------------|-----------------|
|              |          | 1000 | 500 | (mm) | (mm) |
| Honey Sample | E. coli  | 8.00 ± 3.46 | 8.67 ± 3.21 | 0.00 | 26.33 ± 10.02 |
| 1            | S. aureus| 15.17 ± 4.91 | 9.00 ± 0.00 | 0.00 | 33.67 ± 2.31 |
| Honey Sample | E. coli  | 15.00 ± 3.61 | 12.67 ± 1.53 | 0.00 | 32.33 ± 3.21 |
| 2            | S. aureus| 22.67 ± 1.53 | 17.33 ± 6.11 | 0.00 | 34.67 ± 4.51 |
| Honey Sample | E. coli  | 16.33 ± 3.06 | 12.33 ± 2.52 | 0.00 | 38.33 ± 0.58 |
| 3            | S. aureus| 22.67 ± 0.58 | 17.33 ± 4.62 | 0.00 | 33.33 ± 1.53 |

3.3. Anti-diabetic Activity
The IC50 values of anti-diabetic activities of stingless bee honey samples were tabulated in Table 4. All stingless bee honey samples exhibited anti-diabetic activity with honey sample 3>honey sample 2>honey sample 1, and the activity is significantly the same as the positive control, acarbose. In this experiment, alpha–amylase was used to examine the effectiveness of three stingless bee honey sample to reduce sugar from starch. Phenolic acid was reported to be responsible to the anti-diabetic activity [17], and positive correlation was observed between gallic acid content and IC50 value of anti-diabetic. This suggests that the anti-diabetic activity in stingless bee honey sample 2 might be due to the amount of gallic acid in the samples. The present of antioxidants in the honey samples also contribute to the antidiabetic activity by inducing the release of insulin thus lowering blood glucose level [23].

Table 4. The IC 50 values of alpha-amylase inhibition of different samples. Similar alphabets indicate no significant difference at p<0.05, where Tukey test with multiple comparison were performed

| Sample       | IC50 Value ± SD (ug/mL) |
|--------------|-------------------------|
| Honey Sample 1 | 21.84 ± 5.88a           |
| Honey Sample 2 | 16.30 ± 2.98a           |
| Honey Sample 3 | 15.80 ± 1.02a           |
| Acarbose      | 14.56 ± 0.76a           |

3.4. Phytochemical Analysis
Gallic acid has been reported to be present in honey [2], [14]. Table 5 shows the gallic acid content in each of the honey sample, where honey sample 3>honey sample 2>honey sample 1. In this study positive correlation was observed, where honey with high antioxidant and high antibacterial activities contain high amount of gallic acids suggesting gallic acid might be responsible for the activities. It is also possible that the activities were due to synergistic effects of other compounds, such as quercetin [24].
Table 5. The amount of concentration (μg/mL) of gallic acid compound in stingless bee honey samples

| Honey Sample | Concentration of gallic acid in sample (μg/mL) |
|--------------|---------------------------------------------|
| 1            | 29.35                                       |
| 2            | 51.04                                       |
| 3            | 61.17                                       |

3.5. Air Pollution Index of Honey’s Origin

The ambient air quality measurement in Malaysia is described in terms of Air Pollutant Index (API), as shown in Table 6. The geographical origin of honey sample 2 has been showed to have the lowest of air pollutant index (API) at 30.43 ± 7.74 μg/m$^3$ followed by honey sample 3 and honey sample 1 with 51.14 ±10.61 μg/m$^3$ and 52.43 ± 7.89 μg/m$^3$ respectively.

Table 6. The Air Pollution Index (API) of the origin of honey in one week before the day harvested.

| Air Pollution Index (API) (μg/m$^3$) | Honey sample/Location                  |
|-------------------------------------|---------------------------------------|
|                                      | Kulim, Kedah (Sample 1)               |
|                                      | Tg. Malim, Perak (Sample 2)           |
|                                      | Kuala Selangor, Selangor (Sample 3)   |
| 1                                   | 35.00                                 |
| 2                                   | 54.00                                 |
| 3                                   | 55.00                                 |
| 4                                   | 54.00                                 |
| 5                                   | 54.00                                 |
| 6                                   | 59.00                                 |
| 7                                   | 56.00                                 |
| Mean ± SD                           | 52.43 ± 7.89                          |
|                                      | 30.43 ± 7.74                          |
|                                      | 51.14 ± 10.61                         |

4. Conclusion

As conclusion, the biological activities of honey sample (antioxidant, antibacterial, antidiabetic) and gallic acid content seem to be influenced by the air condition of the geographical origin of the honey. The highest antioxidant activity was shown by stingless bee honey samples 2. Honey sample 3 has high antibacterial and anti-diabetic activities in comparison with other honey samples. The gallic acid content of honey sample 3 also the highest. Thus, stingless bee honey originated from environment with a low API index has high quality in terms of biological activity and gallic acid content.

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