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Effect of Thermal Treatment on Kelulut Honey Towards the Physicochemical, Antioxidant and Antimicrobial Properties

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ABSTRACT

Heat treatment on commercial honey could deteriorate its quality associated with physicochemical and bioactive capacities. In this study, the effects of thermal treatment (50 °C, 75 °C and 90 °C) on the physicochemical properties (i.e., pH, colour intensity), total phenolic content and total flavonoid content were investigated on the Kelulut honey. The results revealed a significant increase in TFC (0.154 mg QE/g honey) for the heat-treated Kelulut honey compared to the control (0.085 mg QE/g honey). The antioxidant activity of the heat-treated honey revealed an increase in 2, 2- Diphenyl-1-picrylhydrazyl levels by 42%, while the ferric reducing antioxidant power levels were reduced significantly by 22.4% compared to the untreated honey. The antimicrobial activities of heat-treated honey declined against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Salmonella typhi* bacteria at 75 °C and 90 °C. Based on the effects observed in the bioactive capacities of the heat-treated honey, it is therefore recommended to minimize thermal treatment on the honey during the processing to maintain its natural nutritional quality and benefit consumers.

Keywords: Antibacterial, antioxidant, stingless bee honey, thermal treatment

INTRODUCTION

Honey is widely used as a natural sweetener in food and beverage products. Due to its vast nutritional value, honey is also used because of its medicinal properties. Honey is a viscous solution primarily consisting of carbohydrates, proteins, vitamins, minerals, organic acid, and phenolic compounds (Rao *et al*., 2016). Approximately 100 tonnes of honey are consumed yearly in Malaysia, especially from genus *Apis* sp. and *Trigona* sp. (Ismail, 2014). In Malaysia, stingless bee honey is commonly referred to as Kelulut honey. There are many different species of stingless bee honey, namely *Heterotrigona erythrogastra*, *Tetragonula biroi* and *Heterotrigona itama* (Sahlan *et al*., 2019). Kelulut honey has higher antioxidant and antimicrobial properties compared to Tualang honey (Sulaiman & Sarbon, 2020). Interestingly, Kelulut honey has been known for its anti-inflammatory (Miyata *et al*., 2019), antiangiogenic (Iqbal *et al*., 2019), wound healing (Abd Jalil *et al*., 2017), antidiabetic (Ali *et al*., 2020), anticancer (Diva *et al*., 2019), antioxidant (Chan *et al*., 2017) and antimicrobial (Ngaini *et al*., 2021b) properties. These health benefits are possessed by honey because it consists of active phenolic acids, flavonoids, enzymes, ascorbic acid, protein, and carotenoid groups (Froschle *et al*., 2018). The honey composition and the capacity of bioactive properties are based on the nectar source, seasonal changes, environmental conditions, and processing methods (Ngaini *et al*., 2021a).

Honey is typically consumed fresh from the nest. Nevertheless, heating is applied in industries to delay the crystallization process (Mahnot *et al*., 2019), lowering its water content, avoiding fermentation (Subramanian *et al*., 2007), destroying yeast present in honey and lowering viscosity to facilitate filling (Turhan *et al*., 2008). The heat treatment may influence the physicochemical properties of honey (Sulaiman & Sarbon, 2020) for example acidity, pH, color intensity and sugar composition (Boussaid *et al*., 2018). The treatments are often essentially being assessed to meet the standard requirement and parameters set by the honey industry. As honey

possesses many nutritional benefits, the impact of heat treatment on the antioxidant and antimicrobial activities of honey has become a great concern to consumers. Treating honey to a high temperature exceeding 90 °C could produce toxic substances such as 5-hydroxymethylfurfural (5-HMF) and its derivatives from the Maillard reaction (Subramanian *et al*., 2007; Shapla *et al*., 2018). The time of heat treatment also has an effect on the biological activities of honey (Mahnot *et al*., 2019) but the study which focuses on the heat treatment timeframe on Kelulut honey is still limited.

This study reports the impact of thermal treatment on Kelulut honey at temperatures 50, 75 and 90 °C for 10 minutes, on the physicochemical, antioxidant and antimicrobial properties. The evaluation of the antioxidant properties of the heat-treated honey was performed using 2, 2- Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays, while the broth dilution method was applied to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values for antimicrobial activities. Therefore, this study can provide significant information for food industries by evaluating the effect of thermal on honey products which could benefit the well-being of the consumers.

MATERIALS AND METHODS

Materials

A fresh sample of Kelulut honey (*Heterotrigona itama*) was obtained from a local supplier in Kuching, Sarawak, Malaysia. The sample was stored in an airtight container at 4 °C for further examination. Cultures of bacteria included *Staphylococcus aureus* (ATCC 700698), *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 11775), and *Salmonella typhi* (ATCC 25241) were supplied by Sarawak General Hospital, Kuching. The bacteria cultures were kept in Mueller Hinton Agar at 36 °C for 24 h.

Heating Treatment on Honey

The honey was filtered using Whatman filter paper no. 1 prior to analysis to remove particles. The Kelulut honey samples were heat-treated following method adapted from Sulaiman and Sarbon (2020) with a slight modification. Three test tubes containing 10 ml of honey sample were heated for 10 min, using a water bath at 50, 75 and 90 °C. A nonheat-treated sample was utilized as a control.

Physicochemical Analysis

pH

The pH value of heat-treated honey was obtained by using a pH meter. The pH meter was calibrated at pH 4 and pH 7 standard buffer. The heated and control honey were accurately weighed and diluted in 100 mL of distilled water at 10% (w/v). The results were recorded in triplicates.

Colour intensity

The colour intensity of heat-treated honey was evaluated using the method described by Moniruzzaman *et al*. (2013) with a slight modification. Colour intensity was measured by diluting the honey sample to 10% (w/v) with ultrapure water. Absorbance reading was taken at 450 and 720 nm using Ultraviolet-Visible (UV-VIS) Spectrophotometer (Shimadzu, Japan). Differences in absorbance (Abs 450–Abs 720) were read in mAU. The results were recorded in triplicate.

Biochemical Properties

Total phenolic compound (TPC) analysis

TPC was quantified using the Folin-Ciocalteu method following Azlim Almey *et al*. (2010) with a slight modification. A standard solution of gallic acid (1 mg/ml) was made and used as reference. Standard dilutions were then prepared from 0.02 mg/ml to 0.10 mg/ml. Each heated and controlled honey (100 µl) was added with 0.75 ml of diluted Folin-Ciocalteu reagent, followed by sodium carbonate (0.75 ml, 6% (w/v)). After 90 min of incubation period, the absorbance was measured at 725 nm using a UV-VIS Spectrophotometer (Shimadzu, Japan). The standard calibration curve of gallic acid (0.006 – 0.10 mg/ml) was plotted. The results were recorded in triplicates. The TPC was reported as mg gallic acid (GAE) per gram of honey.

Total flavonoid compound (TFC) analysis

TFC was determined following Zhishen *et al*. (1999) with some modification. Each heat-treated and controlled honey sample (250 µl) was added to sodium nitrite solution (75 µl, 5%). After 6 min, aluminum trichloride (150 µl, 10%) was added to the solution. After that, sodium hydroxide (750 µl, 1 M) was subsequently added to the solution. UV-VIS Spectrophotometer (Shimadzu, Japan) was used to measure the absorbance at 510 nm against quercetin as a reference. The results were recorded in triplicate. The TFC was recorded as mg quercetin (QE) per gram of honey.

Antioxidant Activities

2,2- Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

DPPH activity was evaluated following Shen *et al*. (2010) with slight modification. Each control and heat-treated honey sample (0.5 ml) at various concentrations (3.13, 6.25, 12.50, 25.00, 50.00 and 100 mg/ml) was added to a 1.0 ml of DPPH solution. Ascorbic acid was employed as a positive control. After 30 min incubation in dark environment, the samples were read at 517 nm absorbance using a UV-VIS Spectrophotometer (Shimadzu, Japan). All tests were conducted in triplicates. The DPPH activity was calculated using Eq. (1) (Sulaiman & Sarbon, 2020).

$$\% \text{ inhibition of DPPH scavenging activity} = (A_{control} - A_{sample})/ A_{control} \times 100\% \quad \text{Eq. (1)}$$

Ferric Reducing Antioxidant Power (FRAP)

The assay was conducted following the FRAP Assay Kit (Sigma-Aldrich, USA). The ferrous standard (2 mM) was diluted with FRAP assay buffer to prepare 0, 2, 4, 6, 8 and 10 nmol/well concentrations. The heat-treated honey sample (10 µL) was added to the 190 µl reaction mixture consisting of the FRAP assay buffer, iron (III) chloride solution and FRAP probe. After 60 min of incubation, the absorbance was measured at 594 nm by using UV-VIS Spectrophotometer (Shimadzu, Japan).

Antimicrobial Activities

Minimum inhibitory concentration (MIC)

MIC was evaluated following Wiegand *et al*. (2008) with slight modification. Inoculum colonies were diluted in 4 ml Mueller Hinton broth and adjusted to 0.5 McFarland standard (10^6 Colony Forming Unit (CFU)/ml). A honey solution of 50% (v/v) of 10 ml was prepared by mixing 5 ml honey and 5 ml Mueller Hinton Broth. A series of dilutions of honey solution were prepared (25, 12.5, 6.25 and 3.125%). Tetracycline was utilized as a positive control. All the solutions were inoculated with bacteria cell suspension and kept at 37 °C overnight. After the incubation period, the appearance of turbidity was observed with naked eyes under sufficient lighting. The MIC endpoint was identified with no visible bacteria growth. A confirmation test was performed at 620 nm UV-VIS Spectrophotometer (Shimadzu, Japan).

Minimum bactericidal concentration (MBC)

MBC was measured based on Romainor *et al*. (2014). A 3 µl of the mixture of honey sample and inoculum, which showed non-visible growth of bacteria, was added onto Mueller Hinton Agar. The plates were then incubated at 37 °C overnight. The MBC point was identified as the concentration at which bacteria do not develop.

Statistical Analysis

The results were recorded as mean ± standard deviation except for antimicrobial activities. The one-way analysis of variance test (ANOVA) was performed for the significant analysis of variance, followed by the Tukey post hoc multiple comparison test with a significant, $\alpha = 0.05$. The analysis was conducted by using the SPSS version 24.

RESULTS AND DISCUSSION

Physicochemical Properties of Kelulut Honey

The impact of heat treatment on the pH, colour intensity, phenols, and flavonoids were evaluated after heating at 50, 75 and 90 °C for 10 min. The pH values of heat-treated Kelulut honey are shown in Table 1. pH is one of the crucial physicochemical properties that indicates the acidity that may affect the stability, texture and life span of honey (Froschle *et al*., 2018). The pH of the honey after heating at 50, 75 and 90 °C was within the range of 3.60 to 3.69. The pH was only increased by 2% at 50 °C and 8%

at 90 °C compared to the control honey (pH 3.60). In other words, introducing thermal at 90 °C for 10 min to the honey did not significantly affect the acidity of the Kelulut honey ($p>0.05$). Braghini *et al*. (2019) reported a similar finding on heating the honey, for 60 s at 95 °C showed no significant difference in the pH ($p>0.05$).

Based on Table 1, the colour intensity of the control honey (0.415 mAU) increased by 16% at 90 °C and the increase was not statistically significant ($p>0.05$). Heating the temperature at 90 °C for only 10 min, helped to maintain the honey's colour intensity. The unchanged colour would benefit the market as consumers prefer not to buy or consume darkened colour honey. The heating time is important because a longer heating duration (30 – 120 min) at 45 °C, could alter the physicochemical properties of the honey and the colour intensity (Chong *et al*., 2017). Nevertheless, the heating may result in the browning of the honey because of the Maillard process, where the sugar condenses with amino acids to form brown pigments and subsequently increases its colour intensity (Singh & Singh, 2018).

Biochemical properties of Kelulut honey

The TPC of heat-treated Kelulut honey is indicated in Table 2. There was no significant change observed in the TPC levels between the control and heat-treated honey. The results of the TPC values suggested that the stability of the phenolic compounds and were not affected by the heat applied to the honey. In other words, the phenolic compounds found in the honey are stable upon heating at 10 min, such as benzoic acid derivatives at the temperature of 150 °C (Lindquist & Yang, 2011), quercetin and kaempferol at the temperature range of 100 – 190 °C (Carciochi *et al*., 2016). The insignificant difference in the TPC was also reported by (Elamine *et al*. (2020) on the stability of the phenolic content in Moroccan Zantaz honey upon heating at 120 °C for 30 min.

The TFC of the Kelulut honey increased slightly upon heating at 50 – 90 °C ($p<0.05$). The increase could be due to the possible degradation of the flavonoids such as catechin, myricetin, and naringenin released from the honey's specific bond (Moniruzzaman *et al*., 2014; Sulaiman & Sarbon, 2020). The high flavonoid content resulted in a higher reaction with the aluminium chloride reagent, which increased the honey's TFC values. The total flavonoids of Kelulut honey (8.5 – 15.4 mg QE/100 g) in the present study are comparable to the Romanian honey in the range of 0.91 – 28.25 mg QE/100 g (Al *et al*., 2009). Sulaiman and Sarbon (2020) also reported the increase of flavonoid content in Kelulut honey at 90 °C. However, the study did not indicate the heating time of the honey.

Apart from flavonoids, other chemical compounds were also reported to increase due to heat treatment in honey by previous studies. Using liquid chromatography, α-dicarbonyl compounds which are the key intermediates in the Maillard process were found to increase significantly in the heated acacia honey, compared to the unheated honey (Yan *et al*., 2019). Furthermore, the amino acid compounds were also found to be increased in Tualang, Gelam and floral honey after thermal treatment at 90 °C for 30 min (Chua *et al*., 2014).

Table 1. pH and colour intensity of Kelulut honey treated at three different temperatures (n = 3)

Parameter	Control	50 °C	75 °C	90 °C
pH	3.60 ± 0.02	3.62 ± 0.04	3.64 ± 0.01	3.68 ± 0.03
Colour intensity	0.415 ± 0.012	0.438 ± 0.022	0.459 ± 0.018	0.482 ± 0.018

Table 2. TPC and TFC of Kelulut honey treated at three different temperatures (n = 3)

Parameter	Control	50 °C	75 °C	90 °C
TPC (mg GAE/g honey)	0.145 ± 0.020[a]	0.157 ± 0.013[a]	0.171 ± 0.010[a]	0.191 ± 0.008[a]
TFC (mg QE/g honey)	0.085 ± 0.006[a]	0.123 ± 0.001[b]	0.131 ± 0.001[b]	0.154 ± 0.010[b]

*Value with the different superscript (a-b) within the row was significantly different ($p<0.05$)

Antioxidant Activities of Kelulut honey

Flavonoids are usually involved in antioxidant activity in scavenging free radicals by neutralizing the reactive oxygen (Nijveldt *et al*., 2001; Chan *et al*., 2017). The antioxidant activities of the tested honey were investigated by using DPPH at the concentration of 100 mg/ml, as depicted in Table 3. The antioxidant activities slightly increased after the honey was heat-treated at 50 °C compared to the control. By increasing the temperature to 75 °C and 90 °C, the antioxidant activities using DPPH significantly increased from 68.74% to 89.04% and 97.78% at 75 °C and 90 °C, respectively ($p<0.05$).

The increase in the antioxidant activities using DPPH correlated with the increase in the TFC of the Kelulut honey when the temperature was increased (Sulaiman & Sarbon, 2020). The active compounds, namely flavonoids, are believed to loosen up, resulting in increased antioxidant activities based on the DPPH values (Šarić *et al*., 2013). The antioxidant capacity of water-soluble antioxidants was also supported by FRAP assay to determine the total antioxidant activity (Zarei *et al*., 2019). In contrast to DPPH, the FRAP assay showed a significant decrease following the heat treatment of Kelulut honey ($p<0.05$). The heat treatment has reduced the ferric ions in FRAP by 22% at 90 °C compared to the control temperature. The thermal degradation of the antioxidant compounds in the honey is believed to reduce the FRAP activity (Braghini *et al*., 2019).

The evaluation of the antioxidant activities using DPPH and FRAP assays in this study was in tandem with the antioxidant activities of chestnut, rhododendron, acacia, and multi-floral honey in Turkey, which was reported from weak to moderate activity (Akgün *et al*., 2021). The heat treatment on the honey has led to Maillard reactions that yielded substances with different antioxidant capacities (Turkmen *et al*., 2006; Braghini *et al*., 2019). Even though some of the natural antioxidants are degraded after the heat treatment of the honey, the non-nutrient antioxidants generated by Maillard reactions can compensate and increase the antioxidants' activity (Nayik & Nanda, 2016).

Antimicrobial Activities of Kelulut Honey

The MIC of the heat treatment on Kelulut honey against *S. aureus, B. cereus, E. coli*, and *S. typhi* are summarized in Table 4. The MIC was conducted to identify the lowest concentration of honey that can inhibit the growth of bacteria. The MIC values of the control and heat-treated Kelulut honey were in the range of 12.5% and 25%. The results indicated that the heat-treated honey remained effective against both tested Gram-positive and Gram-negative bacteria. Kelulut honey treated at 50 °C showed the lowest MIC value of 12.5% and comparable to the controlled honey. The MIC value increased to 25% at 75 °C and 90 °C, which indicates a slight reduction of the antimicrobial activity of the heat-treated honey, in comparison with the control (12.5%). The antimicrobial property of the honey could be due to the synergetic effect of the presence of the non-peroxide compound in the honey (i.e., phenolics and flavonoids) and the acidity and high concentration of sugar (Stojković *et al*., 2020; Ngaini *et al*., 2021a).

Table 3. Antioxidant activities of Kelulut Honey treated at three different temperatures (n = 3)

Antioxidant activity	Control	50°C	75°C	90°C
Free radical scavenging (DPPH) activity (%)	68.74 ± 0.003^{a}	71.41 ± 0.009^{a}	89.04 ± 0.022^{b}	97.78 ± 0.007^{b}
Ferric Reducing Antioxidant Power (FRAP) activity (mM)	0.566 ± 0.025^{a}	0.484 ± 0.002^{b}	0.475 ± 0.003^{b}	0.439 ± 0.010^{b}

*Value with the different superscript (a-b) within the row was significantly different ($p<0.05$)

Table 4. MIC in % (v/v) of Kelulut Honey treated at three different temperatures (n = 3)

Bacteria	Control	50 °C	75 °C	90 °C
S. aureus	12.5	12.5	25	25
B. cereus	12.5	12.5	25	12.5
E. coli	12.5	12.5	25	12.5
S. typhi	12.5	12.5	25	25

The MBC was also evaluated to investigate the ability of the heat-treated Kelulut honey to kill the tested bacteria. Like MIC, the MBC of the control and heat-treated honey at 50 °C showed the lowest MBC value of 25% against all bacteria except *S. typhi* (Table 5). However, as the temperature raised to 75 °C and 90 °C, the MBC value was at 50% against all the tested bacteria. This phenomenon was due to the bee-derived enzyme glucose oxidates that produced hydrogen peroxide becoming inactive as the temperature rises (Almasaudi *et al*., 2017). The heat-treated honey would have lower concentrations of hydrogen peroxide, therefore decreasing the antimicrobial activity. In other words, the heat treatment of honey at 50 °C for 10 min, retains the antimicrobial activity as the control against the bacteria compared to the heat-treated honey at a higher temperature. The properties of the non-peroxide compounds for example the flavonoids and phenolics have also contributed to retaining the antibacterial properties of the heat-treated honey (Onyeka *et al*., 2018).

Table 5. MBC in % (v/v) of Kelulut Honey treated at three different temperatures (n = 3)

Bacteria	Control	50 °C	75 °C	90 °C
S. aureus	25	25	50	50
B. cereus	25	25	50	50
E. coli	25	25	50	50
S. typhi	25	50	50	50

Currently, there is no study has been done on the bacterial resistance of Kelulut honey. The resistance is due to the honey's diverse composition, which causes individual or synergistic effects to prevent resistance (Cooper *et al*., 2010; Almasaudi *et al*., 2017). Unlike conventional antibiotics, natural products derived from honey contain various active components with multiple microbial targets (Chen *et al*., 2012).

CONCLUSION

The heat treatment applied on the Kelulut honey up to 90 °C in 10 min gave no significant changes in the pH, colour intensity, and TPC. Interestingly, the water-soluble antioxidants' TFC and antioxidant activity increased at higher temperatures, which medically benefits consumers. Nevertheless, the antioxidant activity indicated by the FRAP levels was lowered at 90 °C. Similarly, the heat treatment decreased the antimicrobial activity. Therefore, it is essential to consider the effect of heat treatment and reduce whichever feasible to sustain the honey's nutritional values and health benefits to humans.

For further studies, quantitative studies using liquid chromatography and mass spectrophotometer to each control and heat-treated Kelulut honey sample should be conducted to understand the compounds contributing to the changes in antioxidant and antimicrobial activities of the honey.

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