

## CONTENT AND ANTIOXIDANT PROPERTIES OF PROCESSED TUALANG HONEY (AGROMAS®) COLLECTED FROM DIFFERENT REGIONS IN MALAYSIA

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### ABSTRACT

The polyphenol and flavonoid contents of 11 processed Tualang honey samples from different origins were found to range from 305.47 to 419.86 mg/kg and 135.29-165.34 mg/kg, respectively. The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was found to range from 28.48% to 36.94% and the total antioxidant activity as measured by the ferric-reducing/antioxidant power (FRAP) assay was found to range between 273.46 and 292.34  $\mu\text{M Fe(II)/kg}$ , indicating that Tualang honey has good antioxidant properties. All of the investigated parameters were found to be higher than another local honey, the Borneo tropical honey. While the total polyphenol contents and antioxidant capacities of all of the processed Tualang honey samples are high, these amounts may vary with the source, origin and post-harvesting processing. It is concluded that processed Tualang honey is a rich source of antioxidants and can be used as a natural food ingredient and a source of antioxidants in the human diet.

**Keywords:** Tualang honey, Polyphenols, Flavonoids, Antioxidant, DPPH, FRAP

### INTRODUCTION

Honey is a food product that is collected from various plants and processed by honey bees (*Apis mellifera*). In different cultures, honey is highly regarded not only for its nutritional value but also its healing properties and has been used in traditional medicine practices. Recently, honey has been scientifically proven to have functional and biological properties, such as antioxidant, anti-inflammatory, antibacterial, antiviral, antiulcerative, antilipid and anticancer properties<sup>1-7</sup>. These activities can be mainly attributed to the phenolic compounds in the honey, such as the flavonoids, which have antioxidant properties and radical-scavenging activities. These activities vary according to the geographical region where the honey was harvested and their climate<sup>8-10</sup>.

Polyphenols, including flavonoids and phenolic acids, are found in honey and act as free radical scavengers, peroxy-radical scavengers and as metal chelators<sup>11, 12</sup>. The average antioxidant properties of a number of honey samples has been determined and found to be significantly correlated to their polyphenol contents<sup>2</sup>.

The antioxidants that are present in honey include both enzymatic and non-enzymatic substances, as well as more than 150 polyphenolic compounds, including flavonoids, flavonols, phenolic acids, catechins and cinnamic acid derivatives<sup>13, 14</sup>. Many methods for determining the antioxidant activity in honey have been used, including the determination of total polyphenol contents<sup>15</sup>, the use of the reporter 2,2-diphenyl-1-picrylhydrazyl (DPPH) to measure superoxide radical-scavenging activity and the use of the ferric-reducing/antioxidant power (FRAP) assay<sup>2, 16, 17</sup>.

Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products<sup>18</sup>. Assays based on the use of DPPH radicals are among the most popular spectrophotometric methods for determining the antioxidant activity in food, beverages and vegetable extracts<sup>19</sup> because chromogenic radical compounds can directly react with antioxidants. The DPPH scavenging method is also preferred for its simple, rapid, sensitive and reproducible procedure<sup>20</sup>.

In Malaysia, Tualang honey is commonly used both in food and as a medicinal product<sup>21</sup>. Recently, the antibacterial properties of this type of honey have been studied and compared with other types of

honey<sup>22, 23</sup>. Tualang honey has good color intensity and contains polyphenol compounds that possess relatively high antioxidant activities<sup>24</sup>. Current research is now directed towards finding antioxidants from natural sources<sup>25</sup>. In this study, we investigated the total content of polyphenols and flavonoids and measured the antioxidant properties of 11 samples of Tualang honey using several chemical and biochemical assays.

### MATERIALS AND METHODS

#### Samples

Tualang honey (AgroMas®) samples were supplied by FAMA (Federal Agricultural Marketing Authority, Kedah, Malaysia). The honey samples were collected by various authorized honey collectors from 11 different regions in the Kedah Rain Forest in March, 2009. Tualang honey samples were compared to Borneo tropical honey, which is another type of honey found in Malaysia.

Honey can be contaminated with various microorganisms during harvesting and packaging. For storage, the acceptable water content in tropical honey is 20%, and filtration is required to remove coarse particles. To use honey for medical research, a suitable sterilization method, such as gamma irradiation, is highly recommended. The honey samples used in our study were individually filtered, evaporated at 40°C to achieve 20% water content by FAMA and sterilized with gamma irradiation at 25 kGy (conducted at STERILE GAMA, Selangor). They were then packed into aluminum sachets for easy distribution and dosing purposes.

#### Chemicals and reagents

Ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) was purchased from Merck (Darmstadt, Germany). Standards of gallic acid, catechin, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), and the Folin-Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, Mo, USA). All chemicals used in this study were of analytical grade.

#### Determination of the total polyphenol content

The levels of polyphenol compounds in the honey samples were estimated with spectrophotometric determination using a modified Folin-Ciocalteu method<sup>26</sup>. Briefly, 1 ml of properly diluted honey

sample (0.2 g/ml) was mixed with 1 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1 ml of 10% sodium carbonate solution was added to the mixture, and the volume was adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm by a T80 UV/VIS spectrophotometer (ChromoTek GmbH, Germany). Gallic acid was used to calculate the standard curve (20, 40, 60, 80 and 100 µg/ml,  $r^2 = 0.996$ ). Phenolic compound levels were measured in triplicate. The results reported are the mean values  $\pm$  standard deviations, expressed as mg of gallic acid equivalents (GAEs) per kg honey.

#### Determination of the total flavonoid content

The total flavonoid content (TF) in the honey samples was determined according to the colorimetric assay method<sup>27</sup>. Briefly, 1 ml of properly diluted honey (0.2 g/ml) was mixed with 4 ml of distilled water, followed by 0.3 ml (5% w/v) sodium nitrite at baseline. After 5 min, 0.3 ml of (10% w/v) aluminum chloride was added. Six minutes later, 2 ml of a 1 M solution of sodium hydroxide was added, and the volume was immediately increased to 10 ml with the addition of 2.4 ml of distilled water. The mixture was shaken vigorously, and its absorbance was read at 510 nm. A calibration curve was prepared using a standard solution of catechin (20, 40, 60, 80 and 100 µg/ml,  $r^2 = 0.996$ ). The results are expressed as mg of catechin equivalents (CEQ) per kg of honey.

#### DPPH free radical-scavenging activity

The antioxidant properties of Tualang honey were also studied by evaluating the ability to scavenge the DPPH free radical. The measurement was based on the method proposed by Ferreira *et al.*<sup>28</sup>. Briefly, 1 ml of properly diluted honey solution (0.2 g/ml) was mixed with 2.7 ml of methanolic solution containing DPPH radicals (0.024 mg/ml). This mixture was vigorously shaken and left to stand in the dark for 30 min (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm<sup>29</sup>. The radical-scavenging activity (RSA) was calculated as a percentage of the DPPH discoloration using the following equation: % RSA =  $[(A_{DPPH} - A_s) / A_{DPPH}] \times 100$ , where AS is the absorbance of the solution when the sample extract was added at a particular level and ADPPH is the absorbance of the DPPH solution.

#### Ferric-reducing/antioxidant power assay (FRAP assay)

The FRAP assay was performed according to the modified method described by Benzie and Strain<sup>30</sup>. Briefly, 200 µl of properly diluted honey (0.1 g/ml) was mixed with 1.5 ml of FRAP reagent. The reaction mixture was incubated at 37°C for 4 min, and the absorbance was then read at 593 nm against a blank of distilled water. The FRAP reagent was pre-warmed to 37°C and was freshly prepared by mixing 10 volumes of 300 mM/L acetate buffer (pH 3.6) with 1 volume of 10 mmol TPTZ solution in 40 mM/L HCl and 1 volume of 20 mM ferric chloride (FeCl<sub>3</sub>·6H<sub>2</sub>O). A calibration curve was prepared using an aqueous solution of FeSO<sub>4</sub>·7H<sub>2</sub>O (100, 200, 400, 600 and 1000 µM/L). FRAP values are expressed as micromoles of ferrous equivalent gallic acid (Ga) per kg of honey.

#### Statistical analyses

Data were analyzed using SPSS (Statistical Packages for Social Science) 12.0 for Windows (Inc., Chicago, IL). The statistical differences indicated by the superscript letters in Table 1 were obtained using a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference post hoc test, with  $\alpha = 0.05$ . Correlations were determined using a regression curve fit model (Table 2), where phenolics and flavonoids were considered to be independent variables and DPPH scavenging activity and FRAP assays were considered to be dependent variables. The assays were conducted in triplicate, and the results are expressed as mean values and standard deviation (SD).

## RESULTS AND DISCUSSION

#### Phenolic and flavonoid contents

Malaysian Tualang honey was found to contain high total phenolic (TP) (Fig. 1) and flavonoid (Fig. 2) content. The TP content of the 11 Tualang honey samples ranged between 305.47 and 419.86 mg gallic acid equivalent/kg (GAE/kg), while the flavonoid content ranged between 135.29 and 165.34 mg CEQ/kg. These levels are high compared to Borneo tropical honey, which was found to contain only 223.20 GAE/kg and 31.89 CEQ/kg of TP and flavonoids, respectively.

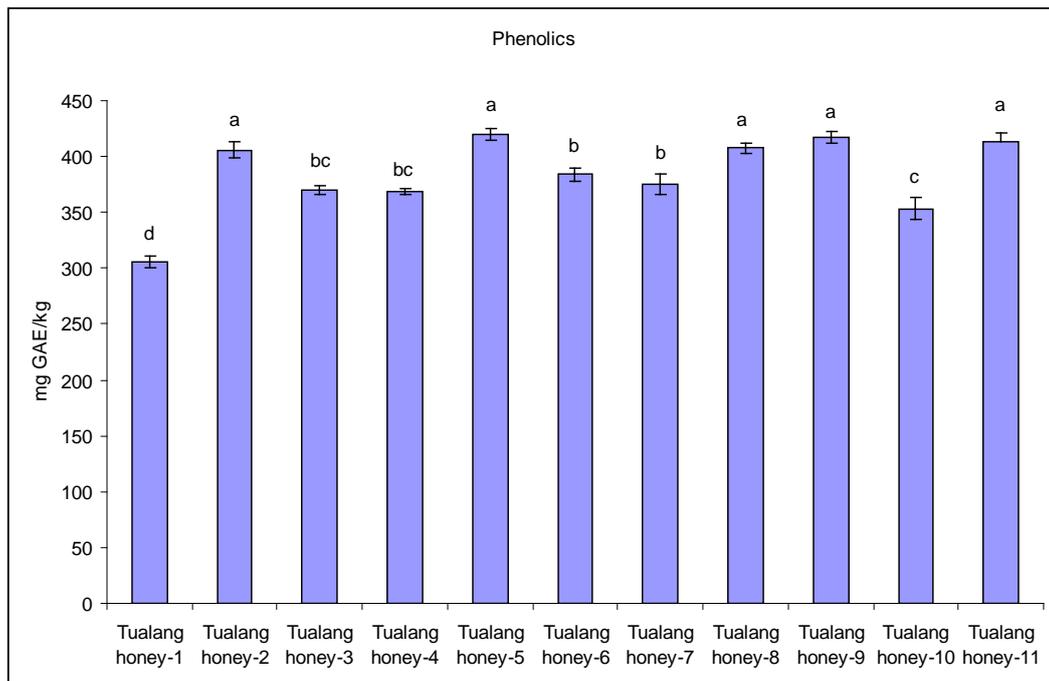


Fig. 1: Phenolics content of eleven Tualang Honey samples (mean  $\pm$  SD; n = 3). In each column different letters mean significant differences ( $p < 0.05$ ); GAE= Gallic acid equivalent.

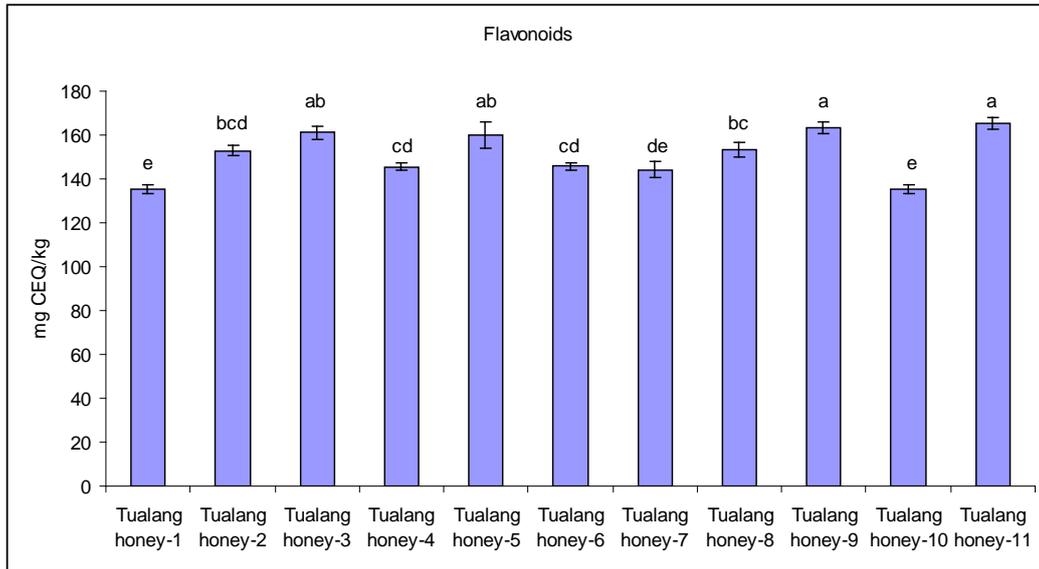


Fig. 2: Flavonoids content of eleven Tualang Honey samples (mean ± SD; n = 3). In each column different letters mean significant differences (p < 0.05); CEQ= Catechin equivalent.

**Antioxidant properties**

Numerous tests have been developed for measuring the antioxidant properties of food and biological samples, but there is still no universal method that can accurately measure the antioxidant capacities of all samples. Clearly, matching the radical source and system characteristics to the mechanisms for generating antioxidant activity is critical for assessing the assay’s ability to measure antioxidant properties<sup>31</sup>.

In this study, two different methods were used to evaluate the antioxidant properties of the Tualang honey samples: the DPPH free radical-scavenging assay and the FRAP assay. DPPH is a stable organic nitrogen radical, and the DPPH radical is commercially available. The DPPH radical-scavenging assay is widely used to evaluate the antioxidant activity of biological samples. The percentage of DPPH inhibition over the duration of the assay reflects the antioxidant activity of the honey tested. The assay duration can vary from 10–20 min up to about 6 h. The FRAP assay is another

commonly used technique to study antioxidant properties. The antioxidant activity of honey can be determined by the ability of the antioxidants in these samples to reduce the ferric ion to ferrous ion in the FRAP reagent, which consists of TPTZ prepared in a sodium acetate buffer at pH 3.6. The reduction of the ferric ion in the FRAP reagent results in the formation of a blue product (ferrous-TPTZ complex), the absorbance of which can be measured at 593 nm<sup>3</sup>.

Using both the DPPH and FRAP assays, we confirmed that all of the Tualang honey samples have high antioxidant activities. The percentage of DPPH radical-scavenging activity ranged from 28.48 to 36.94% (Fig. 3), while the total antioxidant activity measured by the FRAP assay ranged from 273.46 to 292.34 μM Fe(II)/kg (Fig. 4). These levels were higher than those of Borneo tropical honey, the DPPH and FRAP results for which were 25.52% and 221.41 μM Fe(II)/kg, respectively. A good correlation was observed between the TP and flavonoid contents with the antioxidant activity of the honey extracts (Table 2).

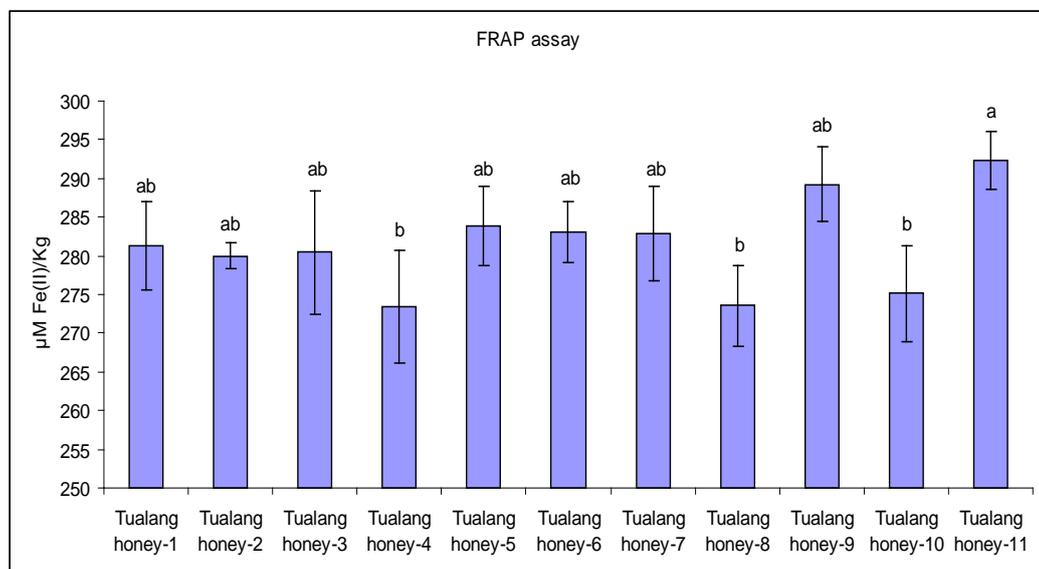


Fig. 3: Results of ferric reducing/antioxidant power assay (FRAP) of eleven Tualang Honey samples (mean ± SD; n = 3). In each column different letters mean significant differences (p < 0.05).

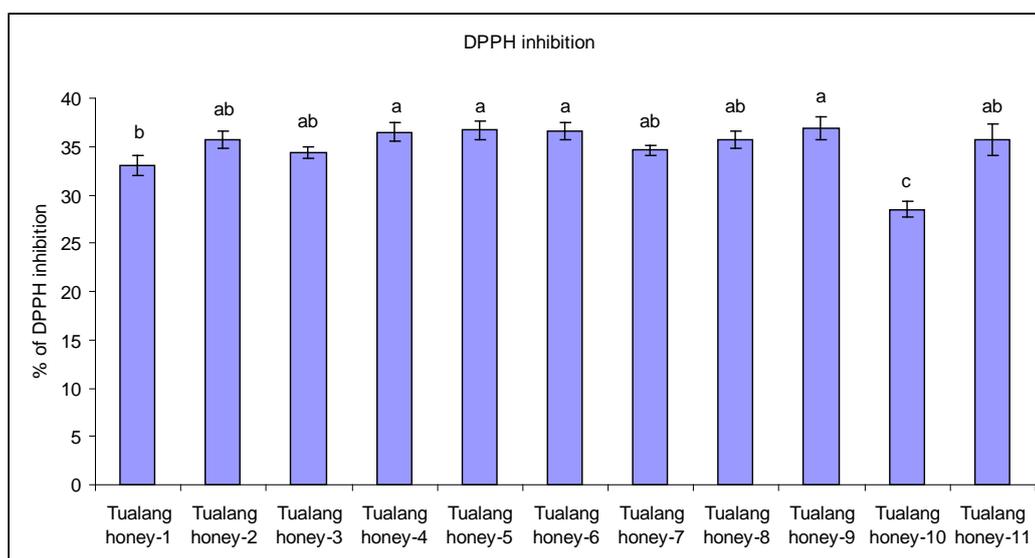


Fig. 4: DPPH radical scavenging activities of eleven Tualang Honey samples (mean  $\pm$  SD; n = 3). In each column different letters mean significant differences ( $p < 0.05$ ); DPPH =1,1-diphenyl-2-picrylhydrazyl.

Table 1: Comparison of phenolic content and antioxidant properties between eleven Tualang honey samples (mean values) and Borneo tropical honey.

Parameters	Mean (n=11) values of Tualang honey	Borneo tropical honey
Phenolics (mg GAE/kg honey)	383.61 $\pm$ 34.53	223.20 $\pm$ 2.89
Flavonoids (mg CEQ/kg honey)	149.77 $\pm$ 12.83	31.89 $\pm$ 1.35
DPPH inhibition (%)	34.93 $\pm$ 2.44	25.52 $\pm$ 1.23
FRAP ( $\mu$ M Fe(II)/Kg)	281.38 $\pm$ 5.99	221.41 $\pm$ 2.52

Table 2: Correlation established between phenolic compounds and antioxidant properties

Chemical parameter	Equation	R <sup>2</sup>	F	p
<b>For Phenolics</b>				
DPPH	Y= 0.0426x+18.5713	0.334	15.53	<0.001
FRAP	Y= .0664x+255.897	0.093	3.18	0.084
<b>For Flavonoids</b>				
DPPH	Y= 0.1356x+14.4294	0.335	15.53	<0.001
FRAP	Y= 0.3238x+232.444	0.219	3.18	0.006

All Tualang honey samples were found to have higher levels of polyphenols than flavonoids. This amount, however, can vary according to the source and post-harvesting processing steps. These results are similar to a previous assessment and to reports of honey from other geographical regions<sup>32, 33</sup>.

Tualang honey 9, which was observed to have a higher value of polyphenols (416.98 mg GAE/kg) and flavonoids (163.29 mg CEQ/kg), exhibited the highest percentage of DPPH radical-scavenging activity (36.94%); Tualang honey 10, which had lower concentrations of phenolics (353.32 mg GAE/kg) and flavonoids (135.46 mg CEQ/kg), exhibited the lowest percentage of DPPH radical scavenging activity (28.58%). On the other hand, the highest antioxidant activity measured by the FRAP assay was 292.34  $\mu$ M Fe(II)/kg for Tualang honey 11, which was found to have 413.57 mg GAE/kg phenolics and 165.34 mg CEQ/kg flavonoids; the lowest value in the FRAP assay was reported for Tualang honey 4 (273.46  $\mu$ M Fe(II)/kg), which was found to have 368.23 mg GAE/kg phenolics and 145.61 mg CEQ/kg flavonoids. On the whole, Table 1 shows that the mean values for all of the parameters are higher for Tualang honey than for Borneo tropical honey (another popular honey in Malaysia). These results clearly demonstrate that processed Tualang honey samples, which have high levels of phenolics and flavonoids, have high antioxidant activity. The use of traditional medicine is widespread and honey represents a large

source of natural antioxidants that might serve as leads for the development of novel drugs<sup>34</sup>.

Several types of compounds can contribute to the antioxidant activity of foods, including carotenoids, ascorbic acid, tocopherols and polyphenol compounds. Table 2 shows that the total phenolic and flavonoid contents correlate with the antioxidant activity. Some studies have concluded that the antioxidant activity can be mainly attributed to the phenolic compounds<sup>35</sup>, and therefore, it is reasonable to expect that the total polyphenol content should be highly correlated with the antioxidant activity<sup>36</sup>.

A strong positive correlation was observed between the total phenolic content and antioxidant activity in all the Tualang honey samples. The correlation between the total phenolic content and DPPH scavenging activity was found to be significant for all Tualang honey samples ( $r^2=0.334$  and  $p=0.000$ ) (Table 2). The results of the FRAP assay, however, were not significantly correlated with the levels of phenolics ( $r^2=0.093$  and  $p=0.084$ ). The higher the content of total phenolics in the honey samples was, the higher was the percentage of DPPH radicals that were quenched. A significant correlation was also observed between the flavonoid content and DPPH scavenging activity ( $r^2=0.335$  and  $p=0.000$ ). The correlation between the flavonoid content and the FRAP results was also significant ( $r^2=0.219$  and  $p=0.006$ ). These results are consistent with previously published data<sup>12, 28</sup> and suggest that the total polyphenol

content and flavonoid content could be good indicators of the antioxidant activity of Tualang honey.

## CONCLUSIONS

It can be concluded that on average, the total polyphenol and flavonoid contents in processed Tualang honey are higher than in Borneo tropical honey. The total phenolic content was found to be higher than that of the flavonoids, but these amounts can vary according to the origin of the honey and the floral sources. The antioxidant properties of Tualang honey, as indicated by the DPPH radical-scavenging activity and the FRAP assay, were found to be higher than those of the Borneo tropical honey. A good correlation was observed between the content of total phenolics and flavonoids and the antioxidant activity of the honey. Therefore, processed Tualang honey can be used as a natural food ingredient as well as a rich source of antioxidants in the human diet.

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