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Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys

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Abstract

The antioxidant activity of two selected Malaysian honeys, as well as their ethyl acetate extracts, were evaluated. The antioxidant activities were determined in terms of their anti radical power (ARP) as assessed by DPPH radical scavenging assay and their total antioxidant power (TAP), as assessed by FRAP assay. Total phenolic content of the extracts was determined according to the Folin-Ciocalteau procedure. The characteristic antioxidant activities showed a marked correlation with the total phenolic contents. These results indicated that honey has antioxidative and radical scavenging properties, which are mainly due to its phenolic content. This is the first report of the antioxidant properties of Malaysian honeys.
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Keywords: Honey; Antioxidant; DPPH; FRAP; Phenolic

1. Introduction

The role of free radicals, such as superoxide radical, hydroxyl radical and others, has been emphasised in a number of diseases, including cancer (Ginter, 1995), cardiovascular disease (Hertog, Feskens, & Hollman, 1993), cataracts (Gerster, 1989), macular degeneration (Vander-Hagen, Yolton, Kaminski, & Yolton, 1993), impaired wound healing (Wana, 1997), gastrointestinal inflammatory diseases (Smirnov, 1994) and other inflammatory processes.

In recent years there has been an increased interest in the application of antioxidants to medical treatment, as information is available linking the development of human diseases to oxidative stress (Zheng & Wang, 2001). Natural food usually contains natural antioxidants that can scavenge free radicals. Small molecule dietary antioxidants, such as vitamin C, vitamin E and carotenoids have generated particular interest as defences against degenerative diseases (Byers & Guerrero, 1995; Kohlmeir & Hastings, 1995; Stampfer & Rimm, 1995). However, some studies have indicated that phenolic substances, such as flavonoids and phenolic acids, are considerably more potent antioxidants than vitamin C and vitamin E (Cao, Sofic, & Prior, 1997; Vinson, Dabbagh, Sevvy, & Jang, 1995); other studies have shown a high correlation between the total antioxidant activity of some fruits and their phenolic contents (Guo, Cao, Sofic, & Prior, 1997; Velioglu, Mazza, Gao, & Oomah, 1998).

The use of honey in the treatment of chronic wounds and diabetic ulcers, cataracts and other eye ailments and peptic ulcers and other gastric ailments has been documented. This beneficial role of honey was partially attributed to its antibacterial activity. However, since some of these diseases have been recognized as being a consequence of free radical damage, it seems that part of the therapeutic role of honey is due to its antioxidant activity. Additionally, the presence of hydrogen peroxide, as well as some minerals (particularly copper and iron), in honey, may lead to the generation of highly reactive hydroxyl radicals as part of the antibacterial system (McCarthy, 1995; Molan, 1992); thus, it is evident that mechanisms must be available in honey to control the formation of these reactive oxygen species and their removal.

Honey contains a number of components known to act as antioxidants; these include vitamin C, vitamin E (Crane, 1975), enzymes such as catalase (Schepartz,

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1966), peroxidase (Ioyrish, 1974) and, phenolic compounds (Ferreres, Ortiz, Silna, Garcia, Vignera et al., 1992). Previous works have indicated that the antioxidant activity of honey varies widely, depending on the floral source (Frankel, Robinson, & Berenbaum, 1998; Chen, Mehta, Berenbaum, Zangerl, & Engeseth, 2000; Gheldof, Wang, & Engesetg, 2001). Available literature indicates that no previous antioxidant property studies have been done on Malaysian honeys; thus the main objective of this work was to evaluate the antioxidant capacity of Malaysian honey, and to evaluate its relationship to the total phenolic contents.

2. Materials and methods

2.1. Materials

Two of the most common Malaysian *Apis mellifera* honeys were used in this study; they are named *Gelam* and *Coconut* honeys, according to their floral sources. All chemicals and reagents used were of analytical grade.

2.2. Extraction and determination of total phenolics in honey

Honey samples were prepared, subjected to base hydrolysis, and extracted with ethyl acetate, as described by Wahdan (1998). Phenolics were then recovered using solid phase extraction (SPE), a modification of the technique used by Seo and Morr (1984) . Briefly, the dry honey extract was redissolved in acidified deionised water (pH 3.5), and the phenolics were adsorbed onto preconditioned isolute C_{18} columns (International Sorbent Tech. Ltd, Hengoed, Mid Glamorgan, UK). The cartridges were preconditioned by sequentially passing 5 ml each of methanol and acidified water (pH 3.5) at a dropwise flow-rate. Phenolic extracts were passed through the preconditioned cartridges at dropwise flow-rate to provide efficient adsorption of the phenolic compounds. The adsorbed phenolics were then eluted from the cartridges by passing 3–5 ml of 25% (v/v) methanol–water solution at a drop wise flow rate. The recovered fractions were combined, dried under nitrogen and subjected to further analysis. Total phenolic contents were determined by the Folin-Ciocalteau procedure, using gallic acid as standard.

2.3. Determination of the free radical-scavenging activity

The method used by Chen et al. (2000) and Frankel et al. (1998) for the measurement of the antioxidant activity of honey was followed in this study. In the presence of an antioxidant, the purple colour of 1,1-diphenyl-2-picrylhydrazyl (DPPH) decays, and the change of absorbency can be followed spectrophotometrically at 517 nm. Briefly, 0.75 ml of honey solution (0.1 g/ml) or of phenolic extracts (0.25–4.0 mg/ml), in warm water, was mixed with 1.5 ml of a 0.09 mg/ml of DPPH (Sigma Chem. Co., St. Louis, USA) in methanol. Each sample was then completely reduced, and the activity was measured spectrophotometrically (Hitachi, U-2000, Japan) at 517 nm, using ascorbic acid (0–0.04 mg/ml) for the calibration curve. Results are given as antioxidant microequivalents (μeq). One antioxidant microequivalent was defined as the ability to reduce one micromole of a pro-oxidant.

2.4. Determination of the total antioxidant power

The ferric reducing/antioxidant power (FRAP) assay, developed by Benzie and Strain (1996) and modified by the same authors in (1999), as a direct method for measuring the total antioxidant power of biological fluids, was adopted in this study. At low pH, reduction of a ferric tripyridyltriazine (Fe^{III}-TPTZ) complex to the ferrous form, which has an intense blue colour, can be monitored by measuring the change in absorption at 593 nm. The reaction is non-specific, in that any half-reaction that has a lower redox potential, under the reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (Fe^{III}) to ferrous (Fe^{II}). Briefly, 300 μl of the working FRAP reagent was pipetted into each well of a 96-well microtitre plate, including reagent blank and standards. Ten micro litres of honey sample (0.1 g/ml), honey extract sample (0.25–4.0 mg/ml), and standard of known (Fe^{II}) value (1000 μM Fe SO_4. $7H_2O$, Merck, Darmstadt, Germany) were added to the corresponding well (10 μl of deionised water was used for the reagent blank). Consequently, the plate was shaken and the absorbance values (A_0) were measured with an automated microplate reader (Power wave $_x$ 340, Bio-Tek Instruments, Inc., Winooski, USA) operated at 593 nm. The plate was then incubated at 37 °C for 4 min, after which the absorbance values (A_4) were measured again. Absorbance change is translated into a FRAP value (μM) by relating the $\Delta A_{593\text{ nm}}$ of test sample to that of a standard solution of known FRAP value (1000 μM Fe^{II}) (Benzie and Strain, 1999), as shown later:

$$\Delta A_{593\text{nm}}\ \text{test sample}/\Delta A_{593\text{nm}}\ \text{standard} \times \text{FRAP value of standard (1000 μM)}$$

2.5. Statistical analysis

All values are expressed as the mean ± standard deviation; significant differences and linear regression analyses were evaluated using the SPSS statistical software.

3. Results

Two of the local Malaysian honeys were used in this study; the antioxidant activity and the total phenolic content were assumed to be different from each other and from other honey samples because of different floral sources. After liquid-liquid extraction, with ethyl acetate and solid phase extraction using C_{18} cartridges, which was used for the first time to recover honey phenolics, the recovered fractions from each 10 g of honey yielded a dry residue of 103.8 ± 7.94 mg (results are the mean of 20 observations ± S.D.). Thus, we assumed that every gramme of honey yielded an extract of nearly 10 mg. The total phenolic contents of Gelam and Coconut honeys were 2.14(±0.129) and 1.56(±0.105) μg/mg extract, respectively, which were significantly different ($P<0.001$). A linear relationship was seen between the different extract concentrations and their total phenolic contents (Fig. 1). Based on these findings, the total phenolic content of Gelam and Coconut honeys can therefore be 21.4 ± 1.29 and 15.6 ± 1.05 μg/g honey, respectively (Fig. 2).

A large variation was found in the antioxidant capacity of both honey types. Gelam honey [36.7(±1.17)$\times10^{-4}$ μequiv] was significantly higher ($P<0.001$) than Coconut honey [22.4(±1.52)$\times10^{-4}$ μequiv] in the free radical scavenging ability. The dose–response curves of the free radical scavenging activities of honey extracts are shown in Fig. 2. In general, among all dilutions tested, the Gelam extracts showed significantly higher ($P<0.001$) capacity than the Coconut extracts. The free radical-scavenging activity of Gelam honey extract was increased in response to increasing extract dose up to 2 mg/ml, at which it reached a steady state and the reaction curve showed a plateau. On the other hand, the free radical scavenging activity of Coconut extract was also increased in response to increasing extract dose, but without reaching an end-point, even by using a dilution of 4 mg/ml. This implied that the free radical-scavenging activity of honey extract is dose-dependent.

The total antioxidant power of Gelam honey [13.45(±0.86)$\times10^2$ μÌ] was found to be significantly higher ($P<0.001$) than that of the Coconut honey [9.61 (±0.64)$\times10^2$ μM]. Gelam extracts also showed significantly higher ($P<0.001$) power than the Coconut extracts, and they showed the same manner as that of the free radical scavenging ability (Fig. 3), indicating that the antioxidant activity of honey extracts are dose-dependent.

To analyse the correlation between the antioxidant activity of honey and their phenolic contents, we plotted the values for the total antioxidant power of honeys against the values of their total phenolic contents (Fig. 4). A significant correlation was found between the total antioxidant power of honeys and their total phenolic contents ($r=0.869$), indicating the role of the phenolic compounds in the antioxidant activity of honey.

4. Discussion

The main objectives of this study were to evaluate the antioxidant capacity of Malaysian honey and to evaluate the relationship between phenolic contents and antioxidant activity. Solid-phase extraction was successfully carried out, for the first time, to recover honey phenolics. It provides a simpler, low cost and speedier technique compared with the use of Amberlite XAD-20 resin, which has been used extensively for the extraction of honey phenolics (Ferreres, Tomas-Barberan, Soler, & Garcia-Viguera, 1994; Martos, Cossentini, Ferreres, & Tomas-Barberan, 1997; Weston, Brocklebank, & Lu, 2000). The total phenolic content of Gelam and

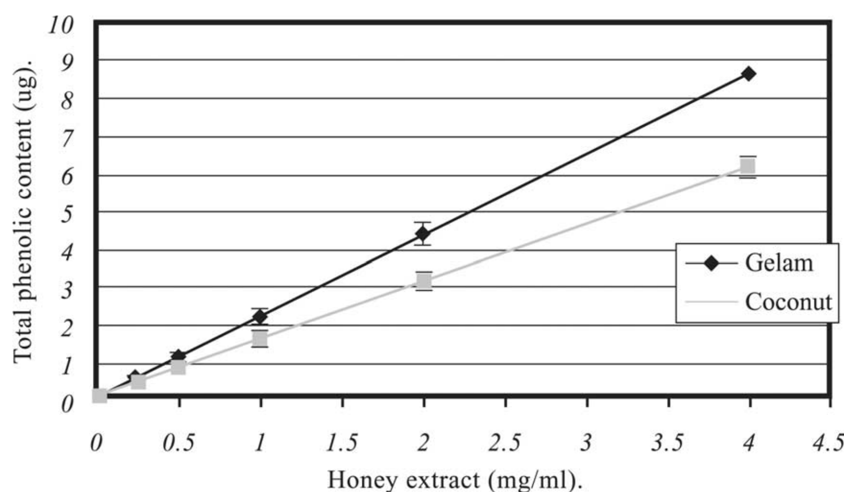

Fig. 1. Total phenolic content of honey extracts (μg/mg extract); data show a linear relationship between extract concentrations and their corresponding total phenolic contents. Results represent means of five determinations (±S.D.).

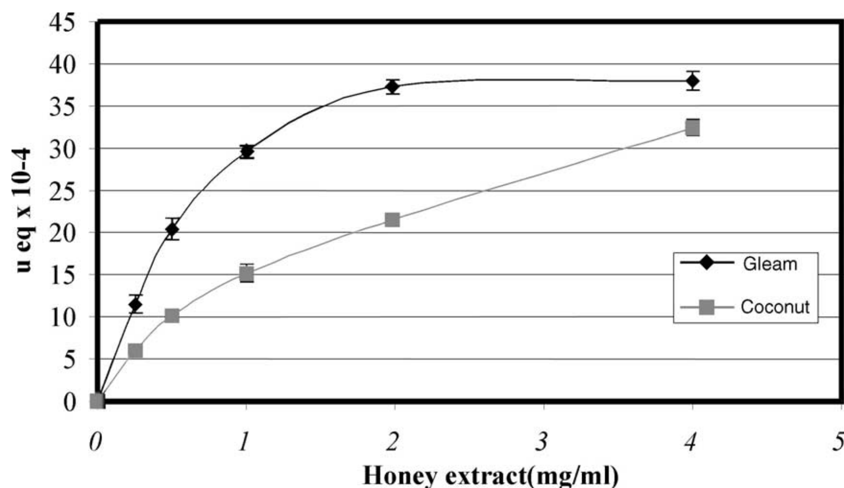

Fig. 2. Dose–response curves for the free radical scavenging activity of Gelam and Coconut honeys extracts (μequiv). Results represent means of five determinations (±S.D.). The activity of Gelam honey extract was significantly higher than that of Coconut honey ($P < 0.001$).

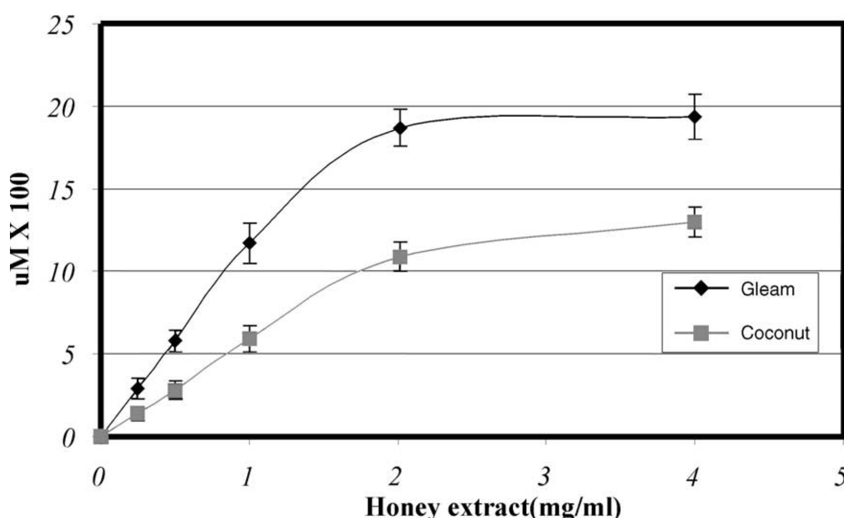

Fig. 3. Dose–response curves for the total antioxidant power of honey extract (μM). Results represent means of five determinations (±S.D.). The activity of Gelam honey extracts was significantly higher than that of Coconut honey ($P < 0.001$).

Coconut honeys was found to be 21.4(±1.29) and 15.6(±1.05) μg/g honey, respectively. Although we used different methods for extraction and determination of total phenolic contents, our results are in agreement with that reported by Ferreres et al. (1992), Gil, Ferreres, Ortiz, Subra, and Tomas-Barberan (1995) and Martos et al. (1997) who found that the total phenolic contents of honeys were between the ranges of 500–2000; 700–2000 and 20–2400 μg/100 g honey, respectively. Since different plants contain different phenolic compounds and show variation in their total phenolic content (Zheng and Wang, 2001), the significant variation between Gelam and Coconut honeys in their total phenolic contents is due to the variation in their floral sources.

The free radical-scavenging activity of *Gelam* honey was 36.7 (±1.17)$\times 10^{-4}$ μequiv, whereas the activity of Coconut honey was 22.4 (±1.52)$\times 10^{-4}$ μequiv, indicating that the reduction potential of Gelam honey is significantly higher ($P < 0.001$) than that of Coconut honey. By comparing these findings with that reported by Frankel et al. (1998) and Chen et al. (2000), who used the same assay, we noted that the free radical scavenging activities of both honeys were higher than the activities of Hawaiian Christmas berry, Tupelo, Sweet clover, Soy bean and Acacia honeys, but less than that of Buckwheat honey. It is noteworthy that this procedure measures only the activity of the water-soluble fraction (Frankel et al., 1998); therefore, these results represent only the activity of the water-soluble antioxidants. Gelam and Coconut honeys were shown to contain 24 and 21% water, respectively (Kamaruddin, 2002), which can partially explain the variation between Gelam and Coconut honeys, and at the same

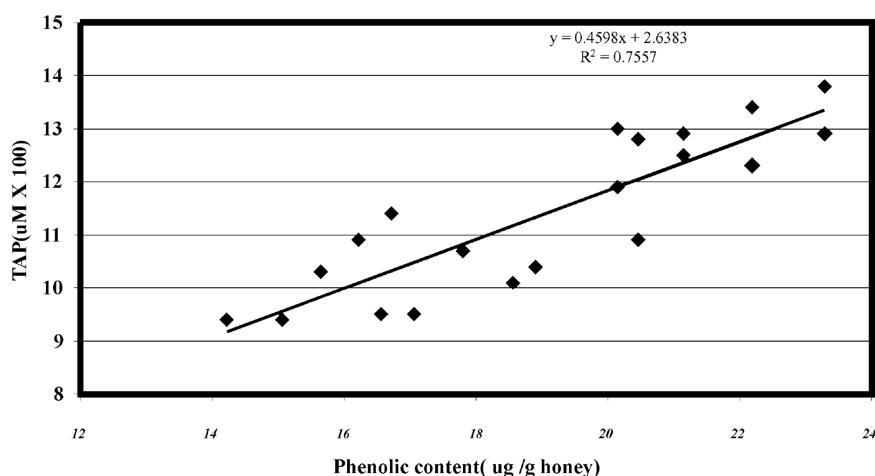

Fig. 4. Correlation between the antioxidant activity of honey and its total phenolic contents. The total phenolic content of honey (μg/g honey) was plotted against the its total antioxidant power (μM). A high correlation was found between the two parameters($r = 0.869$), indicating the responsibility of phenolics about the antioxidant activity of honey.

time is in agreement with the results of Frankel et al. (1998), who found a significant correlation between the water content of honey and its antioxidant activity. Figs. 1 and 2 indicate that the phenolic contents of the two honeys are quantitatively and qualitatively different. It is possible that some of the phenolic compounds present may not be antioxidant agents.

Since the DPPH assay procedure applied here reflects only the activity of water-soluble antioxidants (Frankel et al., 1998), another method was adopted for the evaluation of the total antioxidant activity, i.e. FRAP assay; it is a simple, fast and precise assay, recently developed to measure the total antioxidant power of biological fluids (Benzie & Strain, 1996, 1999). In the present study, the total antioxidant power of *Gelam* honey [$13.5(\pm 0.86) \times 10^2$ μM] was found to be significantly higher ($P < 0.001$) than that of the Coconut honey [$9.61(\pm 0.64) \times 10^2$ μM]. The FRAP assay is used here for the first time to measure the total antioxidant activity of honey, and thus the present results cannot be compared to others. The total antioxidant activities both honeys extracts (Fig. 3) showed the same activity pattern as that of their free radical-scavenging activity. Thus, there are sufficient reasons to believe that the variations in the antioxidant activities of honeys are due to the quantitative and qualitative nature of their phenolic contents. This hypothesis is supported by the findings of other researchers who found variations between different phenolics in terms of their antioxidant activities (Hirano, Sasamoto, Matusmoto, Itakura et al., 2001).

A high correlation was found between the total antioxidant activities of honeys and their total phenolic contents ($r = 0.869$), indicating that phenolics are the components responsible for the antioxidant effects of honey but, obviously, other factors are involved. These might be different phenolic compositions or the presence of non-phenolic antioxidants such as ascorbate, α-tocopherol, and β-carotene. These findings are in agreement with that reported by Wang et al. (1996), Guo et al. (1997) and Velioglu et al. (1998), who found a high correlations between the total antioxidant activities of some fruits and their total phenolic contents.

Gelam honey has been shown to contain some phenolic acids, such as gallic, ferulic, caffeic, benzoic, and cinnamic acids, whereas coconut honey contains gallic, caffeic, and benzoic acids, besides some of other unknown phenolic compounds (Al-Mehdi, 2002). The antioxidant activities of these phenolic acids have been reported previously (Hirano et al., 2001; Ho, Chen, Shi et al., 1992; Laranjinha, Almeida, & Madiera, 1994; Nardini, D'Aquino, Tomassi, Gentili et al., 1995; Yurttas, Schafer, & Warthesen, 2000).

Honey has been used world-wide in the treatment of diseases; however, it seems very likely that the antioxidant mechanism underlies some of the therapeutic effects of honey. Additionally, and from another view of point, honey can play an important role in providing dietary antioxidants in a highly palatable form (Frankel et al., 1998), and can be used as a natural antioxidant to reduce the negative effects of enzymatic browning in fruit and vegetable processing, as recommended by Chen et al. (2000).

In conclusion, the antioxidant activity of Gelam honey was significantly higher than that of Coconut honey, which was due to the difference in their phenolic contents and consequently their floral sources. A high correlation was found between the antioxidant activity of honey and its total phenolic content, indicating that the antioxidant activity of honey is mainly due to its phenolic constituents. The therapeutic value of honey is likely to be in part due to its antioxidant activity. On the other hand, the removal of the reactive hydroxyl radicals, speculated to be generated in honey as part of the

antibacterial system of honey, could therefore be controlled by the antioxidant action of honey. Honey contains both aqueous and lipophilic antioxidants; the interaction between them makes honey an ideal natural antioxidant that can act at different cellular sites.

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