

## Physicochemical analysis and sugar profiling of Acacia honey

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### Abstract

Acacia honey has been traditionally consumed for its nutritional and medicinal values. This study was conducted to investigate the quality of Malaysian Acacia honey obtained from a beekeeper in Johor. Pollen analysis of the honey was subjected to scanning electron microscope (SEM). Free acidity, pH, electric conductivity, sugars, HMF, ash, moisture and antioxidant content of the honey were also determined. Pollen analysis shows the presences of *Robinia pseudoacacia* and an unidentified pollen. Based on the pollen frequency classes, Acacia honey is classified as uni-floral honey. The physicochemical data obtained in this study was within the Codex Alimentarius Standards. Apart from that, Acacia honey has high total phenolic ( $92.04 \pm 0.439$  mg GAE/kg of honey) but low flavonoid ( $27.83 \pm 2.855$  mg QE/kg of honey) contents that responsible for its own sweetness and aroma. In addition, DPPH and FRAP percentage of the samples were concentration dependent. In conclusion, the results of physicochemical analysis of Malaysian uni-floral Acacia honey indicated purity and good quality that meet the standards of natural honey. This study provides additional information for Malaysian honey standard.

**Keywords:** Physicochemical, Sugar profile, Acacia honey.

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### Introduction

Honey is a viscous and supersaturated sugar solution derived from nectar collected and modified by honeybee [1, 2]. It is mainly composed of a complex mixture of carbohydrates, water and other components including phenolic and flavonoids, enzymes, carotenoids, organic acids, and proteins [2]. However, the composition of honey are varies depending on its floral, geographical, and entomological source, seasonal and environmental factors as well as pre and post processing [2].

Acacia honey is produced by *Apis mellifera* that collected the nectar of *Acacia mangium* trees. Acacia honey is traditionally consume for its nutritional and medicinal properties [3]. Although it is not considered a complete food, honey can be a potential supplement for infants, senior citizens, and convalescents as it is easily digestible and ingested directly. Apart from that, it can be used as a sweetener replacement [4].

The quality of honey can be determined based on its physical, sensorial, chemical, and microbiological characteristics [2]. The major criteria for testing quality of honey are moisture content, electrical conductivity (EC), ash content, reducing sugars, free acidity, pH, hydrogen peroxide and hydroxymethylfurfural (HMF) content. The physicochemical characteristics of honey have been extensively studied in Malaysia [2, 5, 6]. However, there is a lack of data on Malaysian honey due to the different physical data obtained by researcher. Therefore, the aim of the present study was to provide additional knowledge on physicochemical analysis of Malaysian Acacia honey obtained from Kluang, Johor as well as its antioxidant and sugar profiling.

### Materials and Methods

#### Honey collection

Acacia honey was obtained from Kluang, Johor (July 2017). The honey was supplied directly by the apiarists. The honey was stored at 4°C prior to analysis.

### **Pollen analysis**

Prior to the analysis, the honey was allowed to reach room temperature and diluted with double distilled water (ddH<sub>2</sub>O) with ratio of 1:5. Honey solution was filtered through PTFE membranes with the aid of filtration system (Merck Millipore, Burlington, United states). Subsequently, the membranes were dried overnight at 37°C followed by pollen morphology analysis under scanning electron microscope (SEM).

Quantification of the pollen was done according to Hamid et al. [7] and Mohamed et al. [8]. Data of pollen count obtained was used to calculate frequency classes of pollen. The frequency classes were classified as “Predominant pollen” (>45% of the total count), “Secondary pollen” (16-45%), “Important minor pollen” (3-15%) and “Minor pollen” (<3%). The pollens were identified with the aid of a pollen atlas.

### **Physicochemical parameters**

The physicochemical analysis of Acacia honey was done in accordance to Harmonised Methods of the International Honey Commission [9]. The data obtained in this study was compared to Codex Alimentarius Commission standard [10] for honey quality.

A pH meter (Mettler Toledo, Ohio, USA) was used to measure the pH of a 10% (w/v) honey solution prepared in ddH<sub>2</sub>O. Free acids in the honey were determined by titrating 10% (w/v) of honey solution using sodium hydroxide (0.1 M w/v) to pH 8.30. Electrical conductivity of 20% (w/v) solution of honey suspended in milli-Q water was measured by using an HI 98311 conductivity meter (Hanna Instruments, Woonsocket, USA) [2]. The results are expressed in mS/cm.

For the measurement of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) level, 20% of honey solution in ddH<sub>2</sub>O was prepared. A peroxide test strip (Merck, Kenilworth, USA) was immersed in the honey solution and the color changes determine the concentration of H<sub>2</sub>O<sub>2</sub> in honey.

The moisture content (%) in honey was determined by refractometer (Atago, Bellevue, USA). Apart from that, the determination of hydroxymethylfurfural (HMF) was done by using the White method [9]. HMF determination was done by dissolving honey (5g) in 25ml ddH<sub>2</sub>O and transferred into 50ml volumetric flask. The solution was mixed with Carrez solution I and II, followed by additional ddH<sub>2</sub>O. The solution was filtered out. The first 10 ml of filtrate was removed. 5ml of the solution was mixed with 5 ml of ddH<sub>2</sub>O (the sample solution). While another 5 ml of the solution was mixed with 5 ml of 0.2% sodium bisulphite solution (the reference solution). Absorbance of the solution was analyzed at 284 and 336 nm.

In order to determine the ash content, a crucible was pre-heated in an electrical furnace which is subsequently cooled and weighted. Honey (5 g) was weighted in pre-heated crucible, covered with olive oil (2 - 5 drops) and heated to 600 °C for 5 hours. The ash was weighted and the data was expressed in g/100g.

### **Identification and quantification of sugar using high-performance liquid chromatography (HPLC)**

Sugar percentage of the honey was determined by using HPLC based on the method published by the International Honey Commission (IHC) [9]. Briefly, honey samples (5g) were dissolved with 40mL of ddH<sub>2</sub>O and mixed with 25 mL methanol. The mixture was transferred into 100 mL volumetric flask, filled with ddH<sub>2</sub>O and filtered using a 0.45 µm nylon membrane filter (Whatman). The samples were then injected (10 µl) into an HPLC system (Agilent, 1200) detected by using Refractive Index (RI) Detector (Agilent, 1200 series). The HPLC column used was a Cosmosil Sugar-D column (4.6mm I.D. x 250mm). Solvents used were of HPLC grade. Table 1 shows condition used to give satisfactory separation.

**Table 1: The condition used for HPLC.**

Instrument setting	Conditions
Flow rate	1.0 mL/min
Mobile phase	Acetonitrile:water (85:20, v/v)
Column and detector temperature	30°C
Sample volume	10 µl

### **Determination of antioxidant properties**

#### **Honey extraction**

Honey extraction [11] was done prior to analysis. Briefly, 20% honey sample was mixed with sodium hydroxide (4N) with 1:1 ratio. The solution was stirred for 4 hours followed by an adjustment to pH 3.5 and transferred into separating funnel. Diethyl acetate (1:1 ratio to total sample solution) and sodium metabisulfite (1g) were added and vortexed to dissolved. The bottom layer was titrated out and mixed again with diethyl

acetate and sodium metabisulfite (the digestion step was repeated for the total of 5). Upper layer was collected and dried in fume hood to obtain the crude sample.

#### DPPH free radical-scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) activity was performed as described by Moniruzzaman et al. [2] with slight modification. Briefly, various concentrations of L-ascorbic acid (10 µL, 0.00 – 0.25 mg/mL) and honey extracts (10 µL, 0 - 3 g/mL) were prepared and mixed with DPPH reagent (90 µL) independently. The plate was incubated for 30 min at room temperature and protected from light. The absorbance was measured at 517 nm by using microplate reader.

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

The antioxidant activity was conducted according to Moniruzzaman et al. [2] method with slight modifications. FRAP reagent was mixed with FeSO<sub>4</sub> (0 - 1mg/mL) or honey extract (3g/mL). The plate was incubated at 37°C and the absorbance was measured by using microplate reader at 593nm after 30 min.

#### Total phenolic content

The concentration of phenolic compounds in honey samples was estimated using a modified spectrophotometric Folin-Ciocalteu method [2]. In a 96-well plate, 50 % of honey solution (80µL) was mixed with 15 % v/v Folin-Ciocalteu (100 µL) and ddH<sub>2</sub>O (10 µL). The plate was incubated in the dark for 5 mins and 0.105 g/mL sodium carbonate (100µL w/v) was added afterwards. After 60 mins of incubation, the absorbance was measured at 756 nm.

#### Total flavonoid content

The total flavonoid content of the honey was measured by using a modified method from Moniruzzaman et al. [2]. Honey solutions (100µL) were mixed with 1.25% w/v sodium nitrate (30µL), 2.5 % w/v aluminum chloride (30 µL), 1 M sodium hydroxide (50 µL) and ddH<sub>2</sub>O (60 µL) in a 96-well plate and analyzed at 510 nm by using a microplate reader.

#### Statistical analysis

The data were reported as mean ± SEM for replicates generated from three independent experiments (n = 3). Statistical analysis was performed by using GraphPad Prism 6. The results were first checked for normality and analyzed using Unpaired T-test. The data is considered statistically significant at p < 0.001.

### Result & Discussion

#### Pollen source, morphology and frequencies classes in Acacia honey

In this study, the presence of pollen in Acacia honey was identified through SEM as shown in Figure 1 (a & b) and Table 2. Two pollen types were identified from the samples comprised of *Robinia pseudoacacia* (Figure 1a) and an unidentified pollen (Figure 1b) in which the pollen counts are 16 and 6, respectively. The morphology of the pollens found in Acacia honey was summarized in Table 2. The pollen size for *Robinia pseudoacacia* was observed to be medium whereas the shape can be identified as sub-prolate with sincolpate aperture. In contrast, the unidentified pollen is small in size with the shape of oblate-spheroidal and inaperture. This data was in accordance with findings reported by Hamid et al. [7] that demonstrated the presence of pollen from *Robinia pseudoacacia* in Acacia honey. However, the unidentified pollen was not reported by Hamid et al. [7]. This is due to the different floral source and geographical origin of the honey.

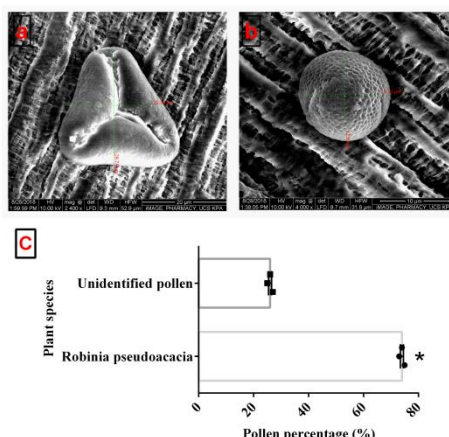
**Table 2: Pollen species found in Acacia honey and its morphology.**

Pollen type	Floral name	Pollen count	Size	P	E	P/E ratio	Shape	Aperture
<b>Fabaceae</b>								
<i>Robinia pseudoacacia</i>	<i>Acacia mangium</i>	16	Medium	28	22	1.27	Sub-prolate	Sincolpate
Unidentified pollen		6	Small	12	13	0.92	Oblate-spheroidal	Inaperture

Note: P, Polar axis and E, Equatorial diameter in µm.

The data from pollen count was used to calculate pollen frequencies and subsequently determine the pollen frequencies classes. In Acacia honey, *Robinia pseudoacacia* pollen was classified as predominant pollen which comprised of 74% ± 1 of pollen species (Figure 1c). Apart from that, unidentified pollen was demonstrated to be secondary pollen (26% ± 1). Honey with pollen species more than 45% is considered as uni-floral honey[7],[8]

and therefore, Acacia honey used in this study is classified as uni-floral honey, corroborated with other findings [1, 4].



**Figure 1: Pollen identified in Acacia honey and the pollen frequencies.** Acacia honey contained (a) *Robinia pseudoacacia* as predominant pollen and (b) unidentified pollen as secondary pollen. \*, indicates statistical significance compared to unidentified pollen using Unpaired T-test,  $p < 0.001$ ,  $n = 3$ .

### Physicochemical analysis

Physicochemical data for this study is summarized in Table 3. In this study, Acacia honey was found to be acidic ( $\text{pH } 3.62 \pm 0.003$ ) and has high free acidity ( $99.3 \pm 0.882 \text{ meq/KG}$ ). The acidity was within the limit ( $\text{pH } 3.4 - 6.1$ ) which indicates freshness and good quality of the honey. The pH value of the honey obtained was similar with those reported in Malaysia [2], Algerian [13], Turkish [14], Brazilian [15], Saudi Arabia [3] and Indian [16]. Honey is naturally acidic and able to inhibit bacteria growth. This is due to the presence of organic acid such as formic acid, oxalic acid, butyric acid, citric acid, 2,3-dihydroxybutanedioic acid, malic acid, pyroglutamic acid, lactic acid, benzoic acid, maleic acid, gluconic acid, isobutyric acid, succinic acid, pyruvic acid,  $\alpha$ -ketoglutaric acid and glycolic acid [3].

Another important parameter for honey quality is the moisture content. The samples have  $19.6 \% \pm 0.11$  of moisture content in which it is within the acceptable level ( $<21\%$ ) by Codex Alimentarius Standards [10]. Low moisture content enables Acacia honey to resist microbial spoilage [2] and helps to maintains the shelf life during storage [17]. Apart from that, the moisture content in honey samples is an indicator to its ability to resist fermentation and granulation during storage [18].

The ash content in honey represents the richness of minerals [19]. The mean of ash content of honey sample was  $0.9 \pm 0.516 \text{ g/100g}$ . According to Codex Alimentarius Standards [10], the limit for ash content is not more than  $1.2 \text{ g/100g}$  which in concurrent with present result.

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**Table 3: Physical parameters of Acacia honey.**

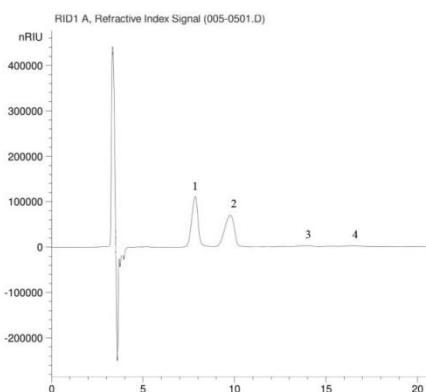
Parameters	Mean	SEM	Range
pH	3.62	0.003	3.62 - 3.63
Free acidity (meq/Kg honey)	99.3	0.882	98 - 101
Moisture content (%)	19.6	0.115	19.4 - 19.6
Ash content (g/100g honey)	0.9	0.516	0.25 - 1.92
EC (mS/cm)	0.813	0.003	0.81 - 0.82
HMF (mg/Kg)	17.5	2.08	13.3 - 19.6
Hydrogen peroxide (mg/L)	1.5	0.5	1 - 2
Fructose (%)	39.9	0.371	40.4 - 39.2
Glucose (%)	30.7	0.285	30.1 - 31.0
Sucrose (%)	4.7	0.088	4.6 - 4.9
Maltose (%)	3.1	0.033	3.0 - 3.1
F/G ratio	1.299	-	-

HMF method was used in this study to provide information regarding the honey quality such as purity, freshness, heat exposure and storage conditions [20]. The HMF concentration in honey sample was demonstrated to be at  $17.5 \text{ mg/Kg} \pm 2.08$ . The concentration of HMF varies between honey. Previous study shows Malaysian Acacia honey have much lower HMF concentration which is  $0.26 \pm 0.2 \text{ mg/Kg}$  [2]. In

contrast, study by Khalil et al. [20] has reported high HMF level in Malaysian tualang honey (128.19 -1131.76 mg/Kg) that have been stored for more than one year. HMF level in fresh honey is usually present in small amounts but tend to increase during processing and prolong storage. Other factors that influence HMF levels are pH, temperature, heating, storage conditions and floral source [21].

Apart from that, the mean value for EC is 0.813 mS/cm  $\pm$  0.003 which is in line with Codex Alimentarius Standards (<1.2 mS/cm). High electrical conductivity reflects high mineral content in honey [3].

H<sub>2</sub>O<sub>2</sub> concentration of the honey was 1.5 mg/L  $\pm$  0.5 which it was within the acceptable value (1 to 2 mg/L). H<sub>2</sub>O<sub>2</sub> is produced by glucose oxidase (GOX) that catalyzed the oxidation of glucose in diluted honey. H<sub>2</sub>O<sub>2</sub> accumulation is highest in 30–50% of honey solutions. Factors influence the level of H<sub>2</sub>O<sub>2</sub> in honey include heat which inhibit GOX, degradation of H<sub>2</sub>O<sub>2</sub> by a catalase enzyme of pollen origin and low levels of GOX in the honey [22].



**Figure 2: HPLC chromatograms of Acacia honey.** Sugar compounds are: 1 (Fructose), 2 (Glucose), 3 (Sucrose) and 4 (Maltose).

The present data showed that sugars are the main composition of Acacia honey. The total sugar content in the samples was 78.44% (Table 3). The chromatogram of reducing sugar was shown in Figure 2. As mentioned in The Council Directive 2001/110 EC mandates [23], the amount of sugars in honey should be more than 60 %. Thus, our findings suggested that sugar compositions of the Acacia honey is within the standard and were similar to other published reports [2, 13, 16]. The fructose and glucose consist of 39.9 %  $\pm$  0.371 and 30.07%  $\pm$  0.285, respectively with fructose/ glucose ratio was 1.299.

The fructose/glucose ratio was within the range of 1.00 to 1.45. High ratio of fructose/glucose enable honey to remains liquid and slow down honey crystallization if fructose/glucose ratio is more than 1.3. However, fructose/glucose ratio below 1.0 can lead to fast crystallization. Apart from that, honey also contains others sugars such as sucrose and maltose as well as other insoluble substances which can influence the crystallization process [24].

The Codex Alimentarius Commission stated that a good quality honey should not contain more than 5 g/100 g sucrose. This is in line with current study whereby shows the sucrose content was 4.7 %  $\pm$  0.088. Maltose only consists of 3.1 %  $\pm$  0.033 from total sugar.

### Antioxidant activity

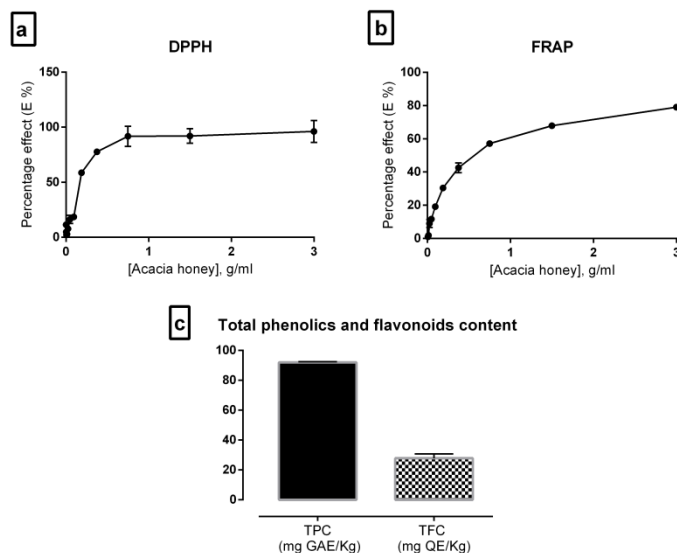
The antioxidant activities of Acacia honey were presented in Figure 3. The ability of antioxidants presence in honey able to reduce DPPH radical and is measured based on discoloration of the DPPH. In contrast, the FRAP assay measures the ability of the sample to reduce the Ferric (Fe<sup>3+</sup>) to ferrous (Fe<sup>2+</sup>).

In this study, activities of the Acacia honey were concentration dependent for both DPPH (Figure 3a) and FRAP assay (Figure 3b). Inhibitory concentration for 50% value measured in Acacia honey for DPPH was 0.1721 mg/ml while FRAP assays showed IC<sub>50</sub> of 0.1572 mg/ml. The IC<sub>50</sub> for the DPPH standard, L-ascorbic acid was found much lower (0.05971 mg/ml) than the honey samples. Apart from that, the FRAP standard, FeSO<sub>4</sub> (0.1839 mg/ml) was found to have similar IC<sub>50</sub> value with Acacia honey sample. Low IC<sub>50</sub> value indicates high scavenging capacity of the honey samples due to the low amount of radical scavenger needed from the honey to reduce DPPH and FRAP [25]. However, the IC<sub>50</sub> for DPPH varies between scientific literatures [25-27]. Similar study conducted in Malaysia (29.846 mg/ml) [25], Africa (45.45 mg/ml) [26] and Croatia (10.53 mg/ml) [27] show Acacia honey require higher concentration in order to inhibit 50% DPPH. Similar pattern was also observed for FRAP assay [28].

The concentration of TPC (Figure 3c) was 92.04 mg GAE/Kg  $\pm$  0.439 (range from 89.18 to 93.31 mg GAE/Kg). This is in line with other study that shows dark-colored honey usually exhibit higher TPC than light-colored honey [29]. In this study, TPC was demonstrated to be higher than in Acacia honey from Italy (55.2  $\pm$  2.8 mg/kg) [26], Slovenia (25.7 to 67.9 mg/kg) [30], Croatia (31.72 to 80.11 mg/kg) [31]. However, the

concentration of TPC in present study was not as high as data reported by Moniruzzaman et al. [32] ( $233.84 \pm 1.52$  mg GAE/kg), Wilczyńska (325.40 mg/kg) [33] and Alzahrani et al. ( $627.56 \pm 44.03$  mg/kg) [34].

TFC of Acacia honey samples ranged from 27.83 - 31.21 mg QE/Kg with the mean of 27.83 mg QE/Kg (Figure 3c). Flavonoids are derived from phenolic compounds that are crucial for the aroma properties and antioxidant capacities of honey [35]. These results were concurrent with study by Abidin et al. [6] that used Acacia honey from the same geographical source. The current data indicates that the phenolic and flavonoids composition in Acacia honey is influenced by the geographical [29] and floral origins [36].



**Figure 3: Antioxidant activities in Acacia honey.** The percentage effect of DPPH (a) and FRAP (b) for the samples was observed to be concentration dependent. In addition, Acacia honey has demonstrated to have high total phenolic ( $92.04 \pm 0.439$  mg GAE/kg of honey) but low flavonoid ( $27.83 \pm 2.855$  mg QE/kg of honey). Mg GAE/Kg, mg Gallic acid equivalents per Kg honey; mg QE/Kg, mg quercetin equivalents per Kg honey.

## Conclusion

The results obtained indicate the purity and freshness of Acacia honey used in this study and storage practices by the beekeepers. Variations in physicochemical properties of the honey compared to other studies was due to the different floral source, geographical area and storage.

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## Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

## Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

## Compliance with ethical standards

The work is compliant with ethical standards.

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