

Physical Properties, Antioxidant Content and Anti-Oxidative Activities of Malaysian Stingless *Kelulut* (*Trigona* spp.) Honey

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Received: October 3, 2017

Accepted: December 6, 2017

Online Published: December 31, 2017

doi:10.5539/jas.v9n13p32

URL: <https://doi.org/10.5539/jas.v9n13p32>

This study was funded by Universiti Kebangsaan Malaysia, under university research grant (GUP-056-2014).

Abstract

Honey produced by stingless bee of *Trigona* spp. is popularly known as *Kelulut* honey (KH) in Malaysia. Even though KH has been increasingly accepted by Malaysians, information relating to its physical and antioxidant properties is still limited. This study aimed to determine the physical properties, antioxidant content and anti-oxidative properties of KH collected from different regions in Peninsular Malaysia. Physical properties of KH including total soluble solids, pH, moisture, ash content, and colour were determined. Antioxidant content namely total phenolics, flavonoids, carotenoids, ascorbic acid equivalent antioxidant content (AEAC) and quercetin equivalent antioxidant content (QEAC) were also quantified. Anti-oxidative potential of KH was assayed using DPPH radical scavenging and FRAP assays. KH has pH of 3.29-3.71, total soluble solids (66.23-73.70 °Brix), 21.40-31.59% moisture and 0.22-0.41% ash content. Colour measurement showed KH from the northern region was lightest ($L = 39.32$) while KH from east coast ($b = -5.06$) and central ($a = 5.65$) regions were more pronounce in blue and red colours. KH from east coast region showed highest values for phenolics (1169.36±51.11 mg GAE/kg), flavonoids (79.13±0.49 mg QE/kg), carotenoids (4.61±0.38 mg/kg), AEAC (146.20±5.56 mg/kg) and QEAC (177.08±5.54 mg/kg). In line with the antioxidant content, honey from the east coast region also had strongest anti-oxidative activities indicated in its lowest IC₅₀ value of DPPH radicals (15.07±1.05 mg/mL) and highest FRAP value (7477.03±48.80 µMFe(II)/kg). The KH collected from different regions showed varied physical and antioxidant attributes which may due to variety source of floral origin.

Keywords: *Trigona*, antioxidant, anti-oxidative, physical properties

1. Introduction

In Malaysia, there are several types of honey produced in this tropical country such as *Tualang* honey, Pineapple honey, *Gelam* honey and *Acacia* honey. Recently, *Kelulut* honey (KH) is increasingly receiving substantial attention from Malaysians due to its distinct flavour and unique sour taste. Unlike other Malaysian honeys, KH is a multifloral honey from stingless bees and it obtained its name from the bees producing it that are locally known as *Kelulut*. *Kelulut* bees are from genus *Trigona*, the largest genus of stingless bees that are indigenous to Neotropics and Indo-Australian regions (Michener, 2000). The bees store their honey in small resin pots near the end of their nests while the honeybees *Apis* spp. store their honey in hexagonal-shaped combs (Kek et al., 2014). *Trigona angustula* has been used widely in Guatemala, Mexico and Venezuela for stomach disorders, cataract and pterygion, respiratory infections and wound healing (Vit et al., 2004). *Trigona carbonaria* that is well known in Australian has been proven to have anti-oxidative and antibacterial properties (Oddo et al., 2008; Boorn et al., 2009).

While market caters a wide range of choices, quality of honey is remained as important issues for consumers. Physical properties are considered as a pivotal domain to determine quality of a honey, affecting the shelf life

and biological activities of honey. Confirming this assertion, international regulatory parties of honey has recommended limit for certain physical parameters. For instance, moisture content of a honey should be not more than 20% (Codex Alimentarius, 2001; EU Council Directive, 2001). Research works has been conducted on Malaysian honey, concluding the moisture content of Malaysian honeys has complied with the limit prescribed (Moniruzzaman et al., 2013a, 2013b). Nevertheless, KH was left out in these studies. This study bridged the gaps of previous studies to provide information pertaining to physical properties of this honey.

Honey has been reported to have more than 200 compounds with sugar as its main components (Escuredo et al., 2013). Phenolic acids, flavonoids and carotenoids are bioactive compounds found in the honey with excellent antioxidant properties. However, these compounds are affected by floral origin, thus causing vast multifarious anti-oxidative properties of different honeys. The notion of natural foods rich in antioxidants are able to scavenge radicals and reduce oxidative stress in human body has been well discerned. Anti-oxidative capacity of honey is believed to be able to alleviate pathological conditions such as cancer, cardiovascular diseases and diabetes. This study aimed to reveal the unexplored antioxidant content and anti-oxidative potential of KH.

2. Methods

2.1 Sample Collection

KH samples were collected at east coast (Pahang), northern (Kedah) and central (Selangor) regions of Peninsular Malaysia. Two batches of samples were collected from each location and each batch of sample was analysed in three replicates in all analyses. The results were expressed as the average \pm standard deviation from six replicates of samples from the same location.

2.2 Physical Analyses of Kelulut Honey

2.2.1 Moisture Content

The moisture content in KH was analysed using table top reflectometer (Abbe, Spain) and the measurements were corrected for the standard temperature of 20 °C with a factor of 0.00023/°C. Wedmore's table was used to determine the moisture content corresponding to the corrected refractive index (AOAC, 1990).

2.2.2 pH

KH sample with concentration of 10% (w/v) was analysed using pH meter (Mettler Toledo, Switzerland).

2.2.3 Total Soluble Solid of Honey

Total soluble solid in KH was tested using handheld refractometer (Atago, Japan). Two drops of concentrated honey was placed and spread on entire surface of the prism of the refractometer and the readings were measured as °Brix.

2.2.4 Ash Content

5.0 g of KH sample was measured inside crucible and ash was obtained by ignition at 550 °C in a furnace (Carbolite Gero, UK) for overnight (AOAC, 1990).

2.2.5 Colour Intensity

The colour intensity in KH was determined using method of Beretta et al. (2005) by absorbance difference of honey at 450 nm and 720 nm using spectrophotometer (Secomam, France). The honey samples were diluted to 50% (w/v) solution with warm (45-50 °C) milli-Q water. The solution was filtered through a 0.45 μ m filter paper. The difference in absorbance was expressed as mAU.

2.2.6 Tristimulus Colour Analysis

The colour measurement in KH was performed using handheld chroma meter (Minolta, Japan). Honey sample (20 g) was poured into glass cell and the reflectance spectrum was measured. The color parameters corresponding to the uniform CIELAB color space in which two color coordinates, a* and b*, as well as lightness, L*, were determined.

2.3 Antioxidant Content

2.3.1 Total Phenolics

The Folin-Ciocalteu assay (Singleton et al., 1999) was used to quantify total phenolic content in KH. Approximately 0.5 mL 10% honey solution was mixed with 2.5 mL 0.2 N Folin-Ciocalteu reagent for 5 min. Subsequently, 2 mL of 7.5% of sodium carbonate solution was added. The mixture was allowed to incubate at room temperature for 2 h. The absorbance of the mixture was then measured at 760 nm using spectrophotometer

(Secomam, France) against methanol as blank. Gallic acid (50-200 mg/L) was used to produce standard curve ($R^2 = 0.987$). The total phenolic content was expressed in mg of gallic acid equivalents (GAE)/kg honey.

2.3.2 Total Flavonoids

Total flavonoid content in KH samples was assayed using the protocol described by Zhishen et al. (1999). A 1 mL 20% honey was mixed with 4 mL distilled water and 0.3 mL sodium nitrite. Following 5 min, 0.3 mL 10% aluminium chloride was added and followed by the addition of 2 mL 1 M sodium hydroxide at six min later. The volume was made up to 10 mL by distilled water. The mixture was shaken thoroughly and the absorbance was read using spectrophotometer (Secomam, France) at wavelength 510 nm. A standard curve was plotted using quercetin solution of concentrations 25-100 $\mu\text{g/mL}$ ($R^2 = 0.987$). The results were expressed as mg quercetin equivalents (QE)/kg honey.

2.3.3 Ascorbic Acid and Quercetin Equivalent Antioxidant Content (AEAC & QEAC)

AEAC and QEAC in KH were performed according to the procedure described by Meda et al. (2005). A 0.75 mL honey in methanol (0.04 g/mL) was mixed with 1.5 mL 0.02 mg/mL methanolic DPPH radical solution. The mixture was left for 15 min at room temperature before the determination of the absorbance using spectrophotometer (Secomam, France) at wavelength 517 nm. The blank sample was made up from 0.75 mL honey solution with 1.5 mL methanol. The AEAC and QEAC were determined using standard curves from ascorbic acid (2.5-10 $\mu\text{g/ml}$) and quercetin (2.5-10 $\mu\text{g/ml}$), respectively. The results were expressed as mg AEAC or mg QEAC per kg honey.

2.3.4 Carotenoid Content

The measurement of carotenoid in KH was performed based on the protocol of Ferreira et al. (2009). A 100 mg honey sample was shaken with mixture of acetone and hexane with the ratio of 4:6 for 1 min. The mixture was filtered with filter paper and the absorbance of the filtrate was determined using spectrophotometer (Secomam, France) at wavelength 454, 505 and 663 nm. Content of β -carotene was obtained using the equation of $0.216A_{663} - 0.304A_{505} + 0.452A_{453}$ while lycopene content was calculated using the equation of $-0.0458A_{663} + 0.372 A_{505} - 0.0806 A_{453}$. The total carotenoid was the sum of β -carotene and lycopene, and all measurements were expressed in mg/100 mL.

2.4 Anti-Oxidative Activities

2.4.1 DPPH Free Radical Scavenging Activity

The scavenging activity of the DPPH radical in KH was conducted based on the procedure of Ferreira et al. (2009). Approximately 0.5 mL 0.2 g/mL honey extract was mixed with 2.7 mL methanolic 0.024 mg/mL DPPH radical solution. The mixture was vigorously shaken and incubated for 15 min prior to the measurement of absorbance at 517 nm. The radical-scavenging activity (RSA) was calculated using the following equation:

$$\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100 \quad (1)$$

where, A_S is the absorbance of the sample with radicals and A_{DPPH} is the absorbance of the DPPH radical solution. IC_{50} was determined as the concentration scavenging 50% of DPPH radicals.

2.4.2 Ferric Reducing Antioxidant Power (FRAP)

This assay was performed using protocol of Benzie and Strain (1999). A 200 μL 0.1 g/mL honey was mixed with 1.5 mL of FRAP reagent prepared from acetate buffer (300 mM/L), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution (10 mM in 40 mM/L HCl), ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) (20 mM) in the ratio of 10:1:1. The mixture was pre-warmed at 37 $^\circ\text{C}$ for 4 min before the measurement of absorbance at wavelength 593 nm using spectrophotometer (Secomam, France). Ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) of concentrations 250-1000 $\mu\text{M/L}$ was used to construct the standard curve ($R^2 = 0.991$). The FRAP values were expressed as $\mu\text{M Fe [II]}$ per kg honey.

2.5 Data Analysis

IBM SPSS version 23 was employed for the statistical analyses. ANOVA with Tukey's Honest Significant Difference (HSD) test was used for comparisons of physical and antioxidant attributes of KH from three different zones. The significant level was set as $\alpha = 0.05$.

3. Results and discussion

3.1 Total Soluble Solids, pH, Moisture and Ash Content of KH

The total soluble solids (66.23-73.70 $^\circ\text{Brix}$) in KH of this study (Table 1) were lower than the values found in previous study (76.2-80.4 $^\circ\text{Brix}$) (Saxena et al., 2010). Total soluble solids can be used as an estimation of sugar

content in which higher total soluble solid value corresponds to higher sweetness (Magwaza & Opara, 2015). Sugars are the main components that are soluble in honey. However, it is not an accurate method to quantify the sugar content as other components in honey such as organic and amino acids may contribute to this reading too.

Table 1. Physical characteristics of KH

Parameters	East coast region honey	Northern region honey	Central region honey
pH	3.71±0.04 ^a	3.71±0.04 ^a	3.29±0.20 ^b
Moisture content (%)	31.59±3.12 ^a	25.10±1.37 ^b	21.40±1.23 ^c
Ash Content (%)	0.41±0.08 ^a	0.23±0.03 ^b	0.22±0.10 ^b
Total Soluble Solids (°Brix)	66.23±0.13 ^a	73.70±0.12 ^b	70.10±0.70 ^c
Color intensity (mAU)	2103.17±3.19 ^a	1200.50±3.21 ^b	1029.00±40.16 ^c
Tristimulus color			
L	35.90 ^a	39.32 ^b	34.57 ^c
a	4.94 ^a	3.98 ^b	5.65 ^c
b	-5.06 ^a	-4.13 ^a	-5.04 ^a

Note. Different letters within the same row indicate the significant difference ($p < 0.05$) among samples.

The pH values of the tested honey samples were in the range of 3.29-3.71. This range was in fair agreement with the values reported in previous studies (3.3-4.7) (de Rodriguez et al., 2004; Silva et al., 2009). Oddo et al. (2008) reported Australian *Trigona carbonaria* honey had a pH of 4.0±0.1, while *Trigona laeviceps* honey from Thailand exhibited a pH of 3.37 (Chanchao, 2009), indicating Malaysian stingless bee honey used in this study had similar pH as stingless bee honeys from other parts of the world. The pH value of honey is influenced by several factors including extraction and storage conditions, while pH itself acts as an attribute to affect texture, stability and shelf-life of honey. This attribute serves as a reliable indicator of microbial stability as most of the bacteria grow in neutral and slightly alkaline medium (Silva et al., 2009). Besides, the acidity gives extra flavor to honey.

Moisture content is an important parameter in determining quality of a honey as high moisture content may reduce the shelf life and microbial stability of a honey. KH in this study were reported to have moisture content of 21.4-31.59%. *Trigona nigra* honey contained 36.2% of moisture (Bijlsma et al., 2006) and *Trigona carbonaria* honey possessed a moisture content of 26.2% (Oddo et al., 2008). The seasonal weather conditions and regional humidity of collection areas are the main factors that influence the moisture content which might be able to explain the great difference of moisture content among honey from different regions of this study. KH had higher moisture content than honey of *Apis* spp. in Malaysia (14.86-19.06%) (Moniruzzaman et al., 2013a, 2013b). Drying process is recommended for this honey following harvesting from the beehive in order to extend the shelf-life of the honey. However, the temperature of drying need to be set low since the phenolics can be destroyed at high temperatures.

The ash content in KH was in the range of 0.22-0.41%, which was within the values reported by previous studies (0.03-1.23) (Malika et al., 2005; Al et al., 2009; Silva et al., 2009). Floral honey should have low ash content. The high dispersion in ash content of honey from different regions may be due to the non-uniformness of techniques used in harvesting and beekeeping process (Finola et al., 2007). Nonetheless, ash content of honey can be varied due to the foraging activities of bees on the floral (de Rodriguez et al., 2004).

3.2 Colour Measurements of KH

ABS₄₅₀ is an indication for the presence of colour pigments such as carotenoids and some flavonoids in honey. However, it could be also contributed by contaminating pigments during the handling, processing and storage of honeys as well by-products of biochemical reactions during honey maturation which did not possess any anti-oxidative properties (Baretta et al., 2005).

KH used in this study had ABS₄₅₀ values at the range of 1029.00-2103.17 mAU. This range was greater than 169.89-740.59 mAU reported by Khalil et al. (2011) and 204-805 mAU by Moniruzzaman et al. (2013b) for various types of Malaysian honey produced by *Apis* spp. honeybees. The higher values of ABS₄₅₀ in KH indicated this honey had higher content color pigments as compared to other Malaysian honeys, giving an added-value to this honey originated from stingless bees. Comparing with honeys from other countries, ABS₄₅₀

of KH was still higher than 70-495 mAU for Slovenian honeys 60 (Bertoncelj et al., 2007), but lower than several types of Italian honeys (25-3413 mAU) (Baretta et al., 2005).

Visual colour of KH were examined in three aspects namely L (lightness), a (redness or greenness) and b for (yellowness or blueness). KH from northern region (L = 39.32) showed the maximum lightness while the central region honey showed the minimum lightness (L = 34.57). The values obtained in this study were lower than those reported for Indian honey (40.96-53.53) (Ahmed et al., 2007) and Slovenian honey (42.12-64.60) (Bertoncelj et al., 2007). The positive a value indicated the presence of red components in the KH whereas the negative b values meant the KH contained blue components.

3.3 Total Phenolics and Flavonoids in KH

Total phenolics found in the KH in this study were ranging from 525.16 to 1169.36 mg GAE/kg (Table 2). KH from the three different regions has a vast difference in their phenolic content. Despite being produced by the same bees (*Trigona* spp.), but the different botanical origin in different regions of Peninsular Malaysia has contributed to this difference. KH is a multifloral honey with no predominant pollen in this honey. The honey was made up from blending of different pollens that were significantly affected by botanical origin, this may clearly explain for the significant difference ($p < 0.05$) for the same type of honey collected from three different regions. Pollen collected from floral origin is the sole contributing source for phenolic content in honey. This was due to the fact that the group of bioactive compounds can only be obtained from plants since bees are unable to secrete any of phenolics from its hypopharyngeal gland.

The phenolic content reported for honey from other geographical origins were 32.59-114.75 mg GAE/100 g for Burkina Fasan honey (Meda et al., 2005), 56.32-246.31 catechin equivalent (CE)/100 g for Yemeni honey (Al-Mamary et al., 2002), 89.98-215.17 mg GAE/kg for Czech honey (Lachman et al., 2010) and 0.24-111.33 mg GAE/100 g for Turkish honey (Silici et al., 2010). The marked difference in reported values has again highlighted the importance of geographical origin that influenced phenolic content of the honey. Comparing with the results from this study, the values of the total phenolics of KH from this study was within the range of previously reported values for honey from various regions mentioned. Meanwhile, the total flavonoids of KH (44.60-79.13 mg QE/kg) were within the range of values (0.91-28.25 mg QE/100 g) reported for Romanian honey from different floral origin (Al et al., 2009). Flavonoids are one of the main classes polyphenols present in pollen collected by honeybees (Kroyer & Hegedus, 2001). This group of bioactive compounds serves as an excellent antioxidant that helps in scavenging free radicals. Flavonoids stabilize and neutralize reactive oxygen species to form a less reactive radical (Nijveldt et al., 2001).

Table 2. Antioxidant content of KH

Parameters	East coast region honey	Northern region honey	Central region honey
TPC (mg GAE/kg)	1169.36±51.11 ^a	720.81±47.50 ^b	525.16±26.38 ^c
TFC (mg QE/kg)	79.13±0.49 ^a	50.14±8.23 ^b	44.60±4.60 ^b
AEAC (mg/kg)	146.20±5.56 ^a	8.26±3.44 ^b	96.64±1.52 ^c
QEAC (mg/kg)	177.08±5.54 ^a	39.75±3.43 ^b	127.74±1.51 ^c
Lycopene (mg/kg)	1.52±0.16 ^a	0.65±0.08 ^b	0.76±0.07 ^c
β-carotene (mg/kg)	3.09±0.23 ^a	1.58±0.12 ^b	1.55±0.06 ^b

Note. Different letters within the same row indicate the significant difference ($p < 0.05$) among samples.

3.4 AEAC, QEAC and Carotenoid Content of KH

The AEAC of KH (8.26-146.20 mg/kg) was lower than those of India (15.1-29.5 mg/100 g) (Saxena et al., 2010), Bangladesh (18.4-34.1 mg/100 g) (Islam et al., 2012) and Algeria (23.68-31.59 mg/100 g) (Khalil et al., 2012) honey. KH from the east coast (177.08±5.54 mg/kg) and central (127.74±1.51 mg/kg) regions contained QEAC within the range of previous study (4.27-33.34 mg/100 g) (Meda et al., 2005). Despite all the honey being produced by *Trigona* spp. bees, both AEAC and QEAC of three honey were significantly different ($p < 0.05$), indicating the important roles of floral source in antioxidant content of honey.

The lycopene content of KH was 0.65-1.52 mg/kg while the β-carotene content was 1.55-3.09 mg/kg. These values were lower than those reported by Ferreira et al. (2009) (β-carotenoids of 8.64-9.49 mg/kg; lycopene of 6.12-6.55 mg/kg) but the total carotenoids in this study (2.23-4.61 mg/kg) was in line with carotenoids of Cuban honey (1.17-5.57 mg/kg) (Alvarez-Suarez et al., 2010).

3.5 Anti-Oxidative Activities of KH

The DPPH assay determines the free radical scavenging activity of honey using stable organic radical 1,1-diphenyl-2-picrylhydrazyl. The IC_{50} , the amount of honey needed to reduce the initial DPPH concentration by 50%, was used as an indicator to compare anti-oxidative capacity of honey in which honey with stronger anti-oxidative potency exhibit lower values (Molyneux, 2004). KH from the east coast region exerted the strongest scavenging activity against DPPH radicals with scavenging percentage of 44.12-79.99% in concentration (10-60 mg/mL) (Figure 1) and the lowest IC_{50} value of 15.07 ± 1.05 mg/mL as compared to 23.98 ± 2.20 mg/mL and 28.86 ± 1.70 mg/mL for honey from the central and northern regions, respectively. Explicitly, the IC_{50} for all three honey were significantly different ($p < 0.01$) and fell within the range of previous studies (1.37-53.80 mg/mL) (Meda et al., 2005; Bertoncelj et al., 2007).

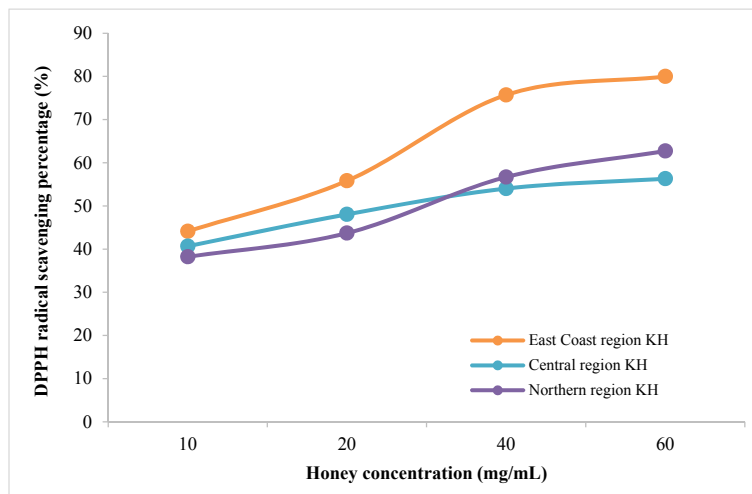


Figure 1. Scavenging activity of DPPH radicals

The FRAP assay estimates the antioxidants and reductants present in honey sample that are able to reduce the Fe^{3+}/Fe^{2+} couple (Beretta et al., 2005). The FRAP values of the tested honey samples were 3630.18-7477.03 μM Fe(II)/kg (Figure 2), and the reducing capacity of KH from east coast was highest followed by KH from northern and central regions. Apparently, the results from this study were higher than those reported for *Tualang*, *Gelam*, Indian forest and pineapple honey (47.92-121.89 μM Fe(II)/100 g) (Kishore et al., 2011). The coincidence of honey from the east coast region having the highest phenolics, flavonoids and FRAP value emphasized contributions of these bioactive compounds in anti-oxidative activity.

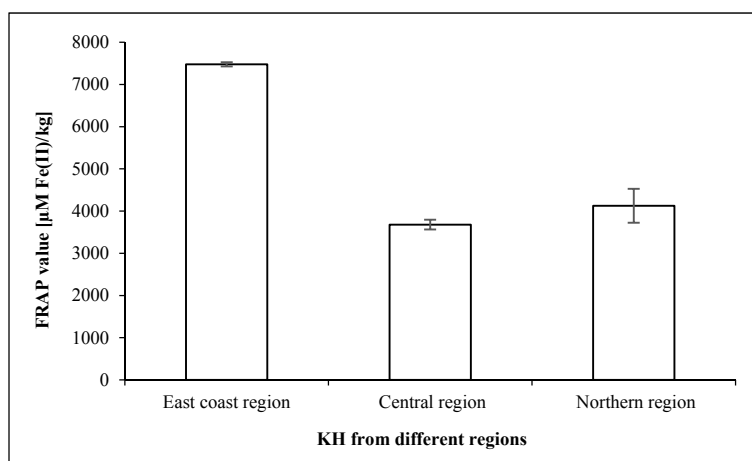


Figure 2. FRAP values of KH

4. Conclusions

The physical properties, antioxidant content and anti-oxidative activities of KH were differed greatly with the geographical origin. Confirming this assertion, the authority governing the production of this honey should generate a grading system as part of the effort to promote this honey. Grading system can be based on the antioxidant content of this honey and consumers can be benefited from this for choosing honey with more antioxidants for their health purpose. Meanwhile, the higher moisture content of the honey might need to be reduced using low temperature drying. This will lower down the moisture content while increase its stability from microorganism and preserve the antioxidant content of KH.

Acknowledgements

The authors would like to acknowledge Universiti Kebangsaan Malaysia for the financial support (University Research Grant, GUP-056-2014) of this study.

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