

Antioxidant Properties of Stingless Bee Honey and Its Effect on the Viability of Lymphoblastoid Cell Line

HAZIRAH H, YASMIN ANUM MY, NORWAHIDAH AK

Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia

ABSTRAK

Penyelidikan madu lebah (kelulut madu) merupakan bidang baru dalam penyelidikan perubatan. Walaupun ia telah digunakan sebagai makanan tradisional dan tambahan selama berabad-abad, pengetahuan mengenai potensi kesihatannya secara saintifik dan terutama pada sifat antioksidannya masih sedikit. Sifat-sifat antioksidan madu diukur dengan menggunakan asai potensi pengurangan ferio, dan mengukur jumlah kandungan fenol dan flavonoid menggunakan spektrofotometri UV. Kandungan jumlah fenolik, jumlah kandungan flavonoid dan potensi antioksidan penurunan ferum madu kelulut Malaysia yang dihasilkan oleh *Trigona* spp. didapati 844.45 mg RE/kg madu, 78.29 mg RE/kg madu dan 1132.66 mM FE/kg madu masing-masing. Penemuan kami menunjukkan korelasi yang kuat antara jumlah fenolik dan kandungan flavonoid dengan potensi antioksidannya pada $R^2 = 0.920$ dan $R^2 = 0.951$. Kesan madu pada daya hidup sel selanjir limfoblastoid (LCL) juga diselidiki. Sel-sel dikulturkan dalam medium RPMI-1640 ditambah dengan 0 - 500 $\mu\text{g/mL}$ madu kelulut selama 24 jam. Daya hidup sel yang diukur menggunakan ujian MTS menunjukkan bahawa suplemen madu meningkatkan daya hidup LCL sehingga 164.64% ($p < 0.01$). Peningkatan ketara dalam daya hidup sel mungkin disebabkan oleh sifat antioksidan madu kelulut.

Kata kunci: antioksidan, flavonoid, lebah tidak bersengat, madu Kelulut, sel selanjir limfoblastoid, fenolik

ABSTRACT

Research on the medical benefit of stingless bee honey (kelulut honey) is rather new although it has been used as traditional food and additive for ages. The primary

Address for correspondence and reprint requests: Norwahidah Abdul Karim. Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel: +603-91459555 Email: wahida2609@gmail.com

objective of our study was to evaluate the antioxidant properties of kelulut honey and its effect on lymphoblastoid cell line. We analysed the antioxidant properties of kelulut honey by ferric reducing antioxidant potential assay, total phenolic and flavonoid contents using UV spectrophotometry. The total phenolic content, total flavonoid content and ferric reducing antioxidant potential of Malaysian kelulut honey produced by *Trigona* spp. were found to be 844.45 mg RE/kg honey, 78.29 mg RE/kg honey and 1132.66 mM FE/kg honey, respectively. Our findings showed a strong correlation between total phenolics and flavanoids contents with its antioxidant potential at $R^2 = 0.920$ and $R^2 = 0.951$, respectively. The effect of honey on cell viability of lymphoblastoid cell line (LCL) was also investigated. The cells were cultured in RPMI-1640 medium supplemented with 0 - 500 µg/mL of kelulut honey for 24 hours. Cell viability was quantitated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS assay showed that honey supplementation boosted the viability of LCL up to 164.64% ($p < 0.01$). The significant increase in cell viability might be modulated by the antioxidant properties of kelulut honey.

Keywords: antioxidant, flavonoid, kelulut honey, lymphoblastoid cell line, phenolic, stingless bee

INTRODUCTION

Honey is a well-known natural food that have antioxidant, anti-inflammatory, anti-diabetic, anti-hypertension and antibacterial properties, just to name a few. Honey is preferred over other supplements as it is a natural product produced by bees. In Malaysia, there are several types of honey including tualang, gelam, nenas, acacia and kelulut honey. Honey possesses anticancer, antibacterial, antimicrobial, anti-ulcer, anti-inflammatory, antinociceptive and antioxidant properties apart from being able to heal wounds and being a potential adjuvant (Hassan & Abdul Karim 2018; Machado De-Melo et al. 2018; Martinotti et al. 2018; Martinotti & Ranzato 2018; Putri Shuhaili et al.

2016; Sawazaki et al. 2018; Stagos et al. 2018).

The main components contributing to these properties of honey are the bioactive constituents in honey including amino acids, organic acids, ascorbic acid, proteins, vitamins, trace elements, and Maillard reaction (Bogdanov et al. 2008; Gheldof et al. 2002; Machado De-Melo et al. 2018). High content of phenolics and flavonoids are mainly responsible for the antioxidant properties of honey, while having low pH and enzymatic glucose oxidation reaction contributes to the antibacterial property of honey (Hassan & Abdul Karim 2018; Putri Shuhaili et al. 2016). In addition, slow absorption of honey leads to the formation of short-chain fatty acid fermentation agents by gut microbiota

(Hassan & Abdul Karim 2018) which are essential for health and wellbeing of the host (LeBlanc et al. 2017; Mohan et al. 2017)

For example, gelam honey contains high level of phenols, proteins, vitamins and amino acid which contribute to its anticancer and antioxidant properties (Goon et al. 2017). Manuka honey also shows antioxidant property as it attenuates H₂O₂-induced oxidative stress in human whole blood cell (Zivkovic et al. 2018). Tualang honey, apart from having anticancer, anti-inflammatory, antioxidant and wound healing properties, it also possess antinociceptive property in human and rats (Abdullah et al. 2015; Aziz et al. 2014). It was reported to improve cognitive function and memory performance (Al-Rahbi et al. 2014; Othman et al. 2011).

Although gelam and tualang honey were used for medical research in the past decades, research on kelulut honey is becoming popular. One unique feature of kelulut honey, as opposed to gelam and tualang honeys, is that the honey was made by stingless bee, locally known as kelulut bee. The *Trigona* spp., is the stingless bees species found throughout tropical regions especially Malaysia Rainforest. Kelulut bees produce kelulut honey, a multifloral honey which is stored in clusters of small resin pots near the extremities of their nests. Honey produced by other bees is stored in hexagonal-shaped combs (Kek et al. 2014). According to the Malaysian Agricultural Research and Development Institute (MARDI), stingless bee honey contains up to ten

times more antioxidants than regular honey and is twice as nutritious (Breen et al. 2016).

Kelulut honey has been proven to possess various pharmacological properties such as anti-inflammatory, antibacterial (Zainol et al. 2013), antiageing, anti-ulcer (Yazan et al. 2018), wound healing (Nordin et al. 2018) and antioxidant properties (Kek et al. 2014; Saiful Yazan et al. 2016). The antioxidant property of kelulut honey has been shown to prevent sperm and testicular oxidative damage in streptozotocin-induced diabetic rats (Budin et al. 2017). The components responsible for the antioxidant properties of honey are likely to be phenolic acids, flavonoids, vitamins, and enzymes (Bogdanov et al. 2008; Erejuwa et al. 2012; Kek et al. 2014). Kek et al. (2014) found that Kelulut honey from three different sources have the highest total phenolic content compared to tualang, gelam, pineapple and borneo honey. Furthermore, honey contains abundant free radical scavengers, which are able to reduce the imbalance between free radical production and antioxidant level (Kishore et al. 2011).

Despite its benefits, little is known about its phenolic acids content, flavonoids content and antioxidant activity. Thus, this study aims to evaluate the total phenolic content, total flavonoid content and ferric reducing antioxidant potential as a measurement of antioxidant activity and the correlation between them. The study also aimed to investigate the effect of kelulut honey on the cell viability of lymphoblastoid cell line (LCL). LCLs

are established by transfecting the B lymphocytes from peripheral blood with Epstein Barr Virus (EBV). They are a promising *in vitro* model system for genetic screening studies, genotype-phenotype correlation studies, a variety of molecular and functional assays related to immunology and cellular biology studies.

MATERIALS AND METHODS

CHEMICAL REAGENTS

2N Folin & Ciocalteu's phenol reagent (FCR) (Sigma-Aldrich, German), rutin (Sigma, USA), $AlCl_3$ (Sigma, USA), Methanol (Merck, German), Sodium acetate, $C_2H_3NaO_2$ anhydrous (Sigma, USA), Acetic acid glacial 100 % (Merck, German), Ferric (III) chloride, $FeCl_3$, Hydrochloric acid, HCl fuming 37 % (System, Malaysia), 2,4,6-tripyridyl-s-triazine, TPTZ (Sigma-Aldrich, German), Ferrous (II) sulphate, $FeSO_4$.

HONEY SAMPLE

Honey sample was purchased from Persatuan Usahawan Lebah Kelulut Darul Naim (DRONESS), Kelantan, Malaysia. The honey was produced by a stingless bee species, *Trigona* spp., and derived from multifloral foraging activity of bees. The honey was collected from the bees from Kelantan, Malaysia in which the beekeepers are the community of the DRONESS. The honey samples were diluted to a concentration of 100 mg/mL in water on the day of assay for Folin-Ciocalteu assay (FCA) and ferric

reducing antioxidant potential (FRAP) and in methanol for total flavonoid content assay and in culture medium to a concentration of 1 mg/mL for cell viability assay.

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic content (TPC) of honey samples was determined by the spectrophotometric Folin-Ciocalteu assay (FCA) as described by Singleton et al. (1999) with some modifications. An amount of 100 μ l of honey solution was mixed with 1 mL of pre-diluted 2N FCR (final concentration 0.2N). The mixture was vortexed for 2 minutes prior to incubation for 20 minutes. The absorbance of the reaction mixture was measured at 750 nm against a distilled water blank using the UV-Visible spectrophotometer (Perkin-Elmer Lambda 25, Waltham, MA, USA). Rutin (0–250 g/mL) was used as a standard chemical for calibration curve preparation. Determinations of TPC were done in triplicates and the total phenolic content was expressed in mg of rutin equivalent (RE) per kg of honey.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content (TFC) of honey samples was determined based on the method described by Isla et al. (2011) with some modifications. An amount of 0.5 mL of honey solution was mixed with 0.5 mL of 2% aluminium chloride ($AlCl_3$) in methanol. A flavonoid-aluminium

complex was formed after 10 min of incubation time at 25°C. The formation of the complex was measured at 415 nm against a methanol blank by using a UV-Visible spectrophotometer (Perkin-Elmer Lambda 25, Waltham, MA, USA). Rutin (0–100 µg/mL) was used as a standard chemical to plot the calibration curve. The TFC was reported as mean value of triplicate assays and expressed as milligram of rutin equivalent (RE) in kg of honey.

FERRIC REDUCING ANTIOXIDANT POTENTIAL (FRAP)

The reducing power of honey samples was determined based on the method described by Benzie and Strain (1996) with some modifications. The principle of this method was based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe³⁺-TPTZ) to its ferrous, coloured form (Fe²⁺-TPTZ) in the presence of antioxidants (Chua et al. 2013; Prior et al. 2005). The FRAP reagent was prepared by mixing 1 mL of a 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, 1 mL of 20 mM FeCl₃, and 10 mL of 0.3 M acetate buffer at the pH of 3.6. The TPTZ solution was prepared fresh and dissolved on 50°C water bath before use. An amount of 40 µL honey solution was well dissolved in 1.2 mL of FRAP reagent, and the absorbance of the reagent mixture was measured spectrophotometrically at 593 nm after incubation for 10 minutes against a distilled water blank using a UV-Visible spectrophotometer (Perkin-Elmer Lambda 25, Waltham, MA, USA). Ferrous (II) sulphate (FeSO₄) (0-2.5

mM) was used as a standard chemical for calibration curve preparation. The FRAP was reported as mean value of triplicate assays and expressed as mM of ferrous (II) sulphate equivalent (FE) in kg of honey.

CELL CULTURE

Lymphoblastoid cell line (LCL) was purchased from Autism Genetic Resource Exchange (AGRE; Los Angeles, CA, USA). The cell line (RUID: HI1315) was derived from healthy male with no documentation of behavioural and neurological disorder.

The cell line was cultured in Rosewell Park Memorial Institute (RPMI) 1640 media (Sigma-Aldrich, German) supplemented with 15 % (v/v) fetal bovine serum (FBS) (Sigma-Aldrich, German), 2 mM L-glutamine (Gibco, USA), 100 U mL⁻¹ penicillin and 100 U mL⁻¹ streptomycin (Gibco, USA) at 37°C / 5% CO₂. The LCL used in this study was within passage 8 to 10.

CELL VIABILITY ASSAY

The cell viability was assessed by using the proliferation media kit, 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, MTS (Promega) as per manufacturer's protocols. Briefly, 50 µL cells (1 × 10⁶ per mL) per well (Niu et al. 2012) were added in a 96-well plate. An amount of 50 µL of kelulut honey was added in each well with various concentrations to achieve the final concentration of 0 - 500 µg/mL of kelulut honey. Three

replicates were performed. The plate was wrapped in aluminium foil and incubated for 24 hours at 37°C/5% CO₂. An amount of 20 µL of MTS solution was added in the 100 µL cultured cell. Aluminium-wrapped plate was further incubated at 37°C/5% CO₂ for 2 hours before reading the absorbance at 490 nm using VERSAmax microplate reader (Molecular Devices).

STATISTICAL ANALYSIS

Data was expressed as mean with standard deviation. The statistical analysis were done using SPSS statistical software version 16. A value of $p < 0.05$ was considered significant following the student's *t* test to determine the significant differences amongst and between the groups.

RESULTS AND DISCUSSION

ANTIOXIDANT PROPERTIES OF KELULUT HONEY

TOTAL PHENOLIC CONTENT

To our knowledge, there is no official method in determining the antioxidant activity in honey samples (Bertoncelj et al. 2007; Chua et al. 2013). It is believed that polyphenols are the radical scavengers due to the presence of high mobility of hydrogens in their molecular structures (Al-Mamary et al. 2002). Thus, determining the total phenolic acid content is crucial as is also strongly correlated with honey antioxidant activity (Beretta et al. 2007; Bertoncelj et al. 2007; Kek et al. 2014).

The TPC of kelulut honey was found to be high (mg RE/kg kelulut honey). TPC measured using FCA is considered to be a fast, inexpensive and simple method to measure the total phenol in complex substance like honey that contains a mixture of components (Chua et al. 2013). According to (Beretta et al. 2005), determining the TPC using FCA is sensitive enough for the total phenol estimation and it includes the polyphenol entitles and other electron-donating antioxidants. In this study, the TPC of the kelulut honey from this company was 844.45 mg RE/kg kelulut honey. This value was higher than that found in New Zealand Manuka honey which is 201 mg RE/kg honey (Jubri et al. 2013). It is difficult to compare the TPC of kelulut honey to other honeys as gallic acid was used to plot the standard curve to obtain the relative value of phenolic content instead of rutin.

TPC measured using FCA is considered to be a fast, inexpensive and simple method to measure the total phenol in complex substance like honey that contains mixture of components (Chua et al. 2013). According to (Beretta et al. 2005), determining the TPC using FCA is sensitive enough for the total phenol estimation and it includes the polyphenol entitles and other electron-donating antioxidants. In this study, the TPC of the kelulut honey from this company is 844.45 mg RE/kg kelulut honey based on the standard curve plotted in Figure 1. This value is higher than that found in New Zealand Manuka honey which is 201 mg RE/kg honey (Jubri et al. 2013).

TOTAL FLAVONOID CONTENT

To date, there are two different spectrophotometric methods in determining the total flavonoid content, which both measures the formation of coloured complex substances quantitatively (Chang et al. 2002). The difference between the two methods is the compound used to react with flavonoid, which one uses aluminium ion (III), usually from aluminium chloride ($AlCl_3$), while the other uses 2,4-dinitrophenylhydrazine (DNP). However, certain flavonoids such as flavones and flavonols could not react with DNP, thus suggesting that the former method is preferred in determining the total flavonoid content (Chang et al. 2002). On top of that, (Iurlina et al. 2009) found that flavonols is the most predominant flavonoid in honey samples. In this study, the TFC of the kelulut honey from this company is 78.29 mg RE/kg kelulut honey.

The TFC from our kelulut honey was higher than that found in Portuguese heather honey by more than 15-fold (between 0.6 to 5 mg RE/kg honey) and almost 4-fold higher than Spanish rosemary honey (between 5 to 20 mg RE/kg honey) (Ferrerres et al. 1994). However, the TFC value was lower

than what was found in tualang honey (185.11 mg RE/kg honey), gelam honey (328.86 mg RE/kg honey) and acacia honey (307.41 mg RE/kg honey) (Chua et al. 2013).

The ratio of TFC/TPC is 0.093. Phenolic compounds have redox properties due to the presence of hydroxyl groups that provide free radical scavenging ability, which allow them to act as antioxidants (Baba & Malik 2015). Thus, the TPC could be used as a basis for rapid screening of antioxidant activity. Flavonoids also use the same basis. Thus rutin, a flavanoid, can be used as the reference standard.

FRAP

The FRAP assay is simple, speedy, inexpensive, and robust (Prior et al. 2005). In this FRAP assay, the amount of iron reduced is correlated to the amount of antioxidants (Pisoschi & Negulescu 2011). It was found that the antioxidant activity of our kelulut honey is 1132.66 mM FE/kg. This value is 5-fold higher than that was found in New Zealand manuka honey (215.71 mM FE/kg honey) (Jubri et al. 2013). Although there are a lot of FRAP assays done on other honeys, comparison

Table 1: Total phenolic content (TPC), total flavonoid content (TFC) and FRAP value of kelulut honey (RE = rutin equivalent; FE = ferrous (II) sulphate equivalent)

Parameter	Kelulut
Total phenolic content	844.45 ± 49.70 mg RE/kg
Total flavonoid content	78.29 ± 28.65 mg RE/kg
FRAP	1132.66 ± 151.93 mM FE/kg

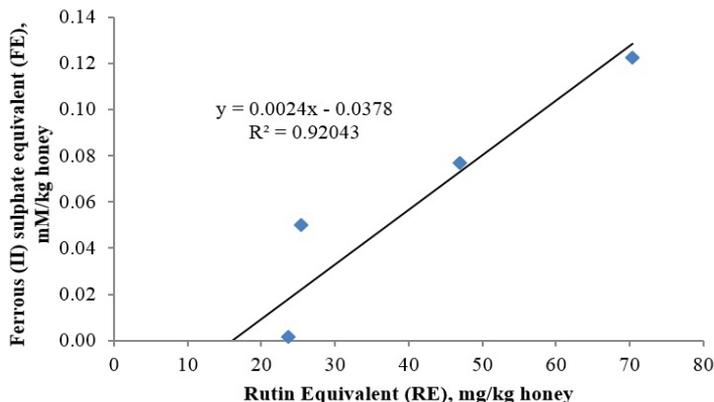


Figure 1: Correlation between total phenolic content and FRAP of Malaysian kelulut honey. Determination was done in triplicates and the mean value was used to calculate the FE and RE values.

between the FRAP value cannot be performed due to similar reasons as the TPC value. Whilst some publications use ferrous (II) sulphate concentration as the reference standard, others also use trolox and ascorbic acid as the reference standard. Lack of standardisation of methods can lead to several orders of magnitude difference in FRAP value, thus its antioxidant capacity.

Table 1 summarises the value of TPC, TFC and FRAP that was found in

Malaysia kelulut honey in this study.

CORRELATION BETWEEN TPC AND FRAP.

The antioxidant property of kelulut honey, determined via the FRAP assay, was found to correlate significantly with the total phenolic content, $R^2 = 0.920$ (Figure 1). This strong correlation supports that polyphenols are the compounds responsible for the antioxidant property of honey.

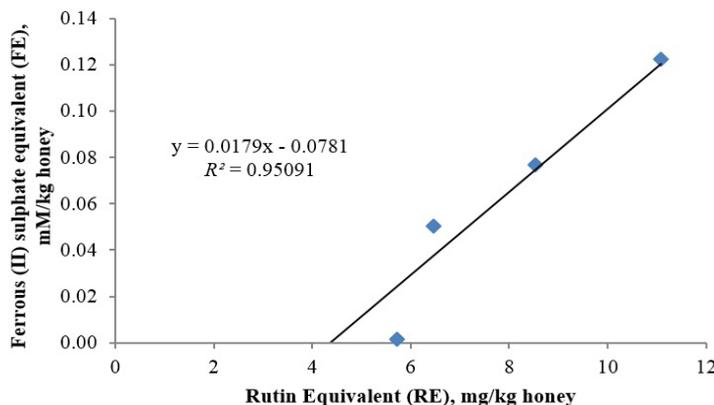


Figure 2: Correlation between total flavonoid content and FRAP of Malaysian kelulut honey. Determination was done in triplicates and the mean value was used to calculate the FE and RE values.

CORRELATION BETWEEN TFC AND FRAP

The correlation between the TFC and the antioxidant property of honey based on FRAP assay then determined as flavonoid contents were found to be the crucial compounds that contributes to the antioxidant properties of honey. Based on Figure 2, the TFC strongly correlated with the FRAP of this Malaysian kelulut honey with high regression coefficient, R^2 of 0.951. This strong correlation suggests that flavonoids could be one of the major contributors for the antioxidant property of honey, apart from phenolics.

From these fast screening methods, FCA, TFCA and FRAP assay, the antioxidant properties of stingless bee honey from DRONESS could be determined. The TPC found could not be compared to other TPC values found in other honey due to the difference in the standard reference used except for New Zealand manuka honey which was found to have lower TPC value. On the other hand, the TFC value of kelulut honey was higher than Portuguese heather honey Spanish rosemary honey although the TFC value is lower than what was found in tualang honey, gelam honey acacia honey. The FRAP value found was higher than the New Zealand manuka honey. Both the phenolic and flavonoid content of honey samples strongly correlated with the antioxidant activity, FRAP ($R^2= 0.920$ and 0.951 , respectively).

Table 2 summarised the TPC, TFC and the antioxidant capacity measured

in different types and sources of honey in comparison with the honey used in this study.

From these fast screening methods, FCA, TFCA and FRAP assay, the antioxidant properties of stingless bee honey from DRONESS could be determined. The TPC found could not be compared to other TPC values found in other honey due to the difference in the standard reference used except for New Zealand manuka honey which was found to have lower TPC value. On the other hand, the TFC value of kelulut honey was higher than Portuguese heather honey Spanish rosemary honey although the TFC value is lower than what was found in tualang honey, gelam honey acacia honey. The FRAP value found was higher than the New Zealand manuka honey. Both the phenolic and flavonoid content of honey samples strongly correlated with the antioxidant activity, FRAP ($R^2= 0.920$ and 0.951 , respectively).

EFFECT OF KELULUT HONEY ON LCL VIABILITY

In this study, the antioxidant property of honey was tested against the viability of LCL via MTS assay. MTS assay is a well-established method for the measurement of cell proliferation in response to growth factors, cytokines, mitogens and nutrients. The principle of the assay is based on the reduction of MTS tetrazolium compound by metabolically active and viable cells through the activity of NAD(P)H-dependent dehydrogenase enzymes to generate a colored

Table 2: The total phenolic content, total flavonoid content and antioxidant property (FRAP) from different types and sources of honey reported previously.

Honey Sources	Honey types	Total phenolic content (mg/kg honey)	Total flavonoid content (mg/kg honey)	Antioxidant activity by FRAP assay (mM FE/kg honey)
Malaysian honey*	Kelulut	844.45 ± 49.70	78.29 ± 28.65	1132.66 ± 151.93
Malaysian honey# [1]	Tualang	589.2	-	-
	Nenas	602.4	-	-
	Brunei	510.4	-	-
	Kelulut	1058.8 ± 2.1	-	-
Malaysian honey [23]	Tualang	251.7 ± 7.9	-	322.1 ± 9.7
Malaysian honey [7]	Tualang	839.6 ± 45.3	504.5 ± 18.3	1218.9 ± 38.7
	Gelam	741.2 ± 25.2	461.1 ± 7.1	1156.1 ± 38.6
Malaysian honey [24]	Gelam	52	6.92	-
Malaysian honey [25]	Gelam (0.4 g/ml)	417.6 ± 8.4	26.4 ± 1.2	689.37 ± 23.6
	Nenas (0.4 g/ml)	216.0 ± 4.5	19.7 ± 2.1	311.4 ± 7.97
Malaysian honey [26]	Tualang	383.79 ± 13.57	49.04 ± 0.53	-
	Gelam	606.17 ± 20.36	183.43 ± 1.70	-
	Acacia	550.00 ± 21.93	115.96 ± 4.69	-
New Zealand honey [17]	Manuka	201.00 ± 35.92	-	215.71 ± 50.00
Croatian honeys ## [27]	Monofloral	42.24	-	82.31
	Heterofloral	58.75	-	157.66
Slovenian honeys** [14]	Acacia	44.8 ± 14.8	-	71.0 ± 10.2
	Lime	83.7 ± 14.3	-	118.8 ± 20.3
	Chestnut	199.9 ± 34.1	-	360.1 ± 66.5
	Fir	241.4 ± 39.5	-	478.5 ± 95.5
	Spruce	217.5 ± 20.6	-	395.3 ± 69.6
	Multifloral	157.3 ± 20.9	-	224.8 ± 24.7
	Forest	233.9 ± 21.7	-	426.4 ± 41.5
Commercial honeys # [16]	Strawberry tree	789.6 ± 13.8	-	1501.4 ± 60.2
	Buckwheat	482.2 ± 2.4	-	800.7 ± 23.8
	Chestnut	211.2 ± 5.5	-	388.6 ± 8.2
	Sulla	106.6 ± 4.6	-	155.2 ± 6.6
	Clover	67.1 ± 5.6	-	72.8 ± 3.0
	Dandelion	102.1 ± 10.0	-	224.4 ± 6.0
	Chicory	158.5 ± 3.8	-	209.5 ± 2.8
	Acacia	55.2 ± 2.8	-	79.5 ± 3.7
	Multifloral	170.4 ± 1.7	-	361.9 ± 10.8
	Honeydew	255.6 ± 7.5	-	772.0 ± 21.5
African tropical	595.2 ± 13.1	-	808.1 ± 18.3	

* denotes honey used in this study; # denotes the highest value amongst honeys tested in the respective study; ** denotes value per 100 g honey and ## denotes mean value amongst honeys tested in the respective study.

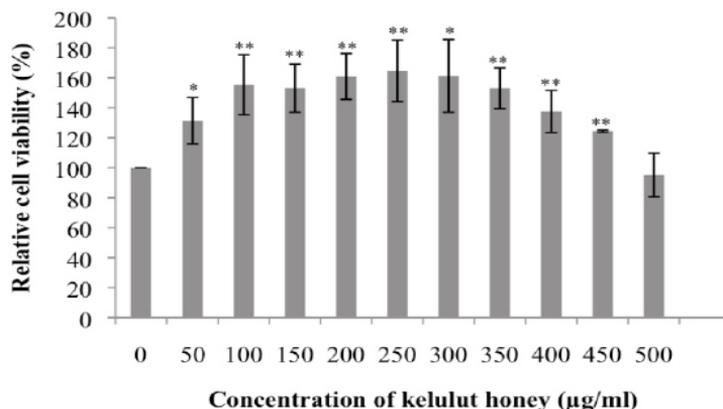


Figure 2: Relative cell viability of kelulut honey-treated LCL determined by MTS assay. Results are expressed as mean + S.D. * denotes $p < 0.05$ while ** denotes $p < 0.01$ compared to respective cell not supplemented with kelulut honey (0 µg/ml) as determined by Student's t test.

formazan product.

In biomedical research, obtaining source of biomaterial from individual for molecular studies has always been a constraint. Isolating peripheral blood mononuclear cells (PBMCs) is an alternative as human peripheral blood is readily accessible and could become the substitute in the absence of access to organs especially the brain. The phenotypes obtained in this model can reflect both genetic and environmental effects on an individual (Morrison et al. 2016).

Generation of LCLs as a surrogate for replacement of isolated PBMCs is getting popular especially in the field of oncology and neurodegenerative diseases. LCLs are established by transfecting the B lymphocytes from peripheral blood with Epstein Barr Virus (EBV). LCLs fulfill the requirement of constant supply of starting material for variety of assays, without the need of re-sampling, relatively easy to prepare and the maintenance is effortless (Hussain & Mulherkar 2012). LCLs provide an unlimited source of

biomolecules such as DNA, RNA and proteins (Sie et al. 2009). They are a promising *in vitro* model system for genetic screening studies (Sie et al. 2009), genotype-phenotype correlation studies, a variety of molecular and functional assays related to immunology and cellular biology studies.

The cell viability assessment of LCL with various honey concentrations, revealed a concentration-dependent effect of honey on cell viability (Figure 3). The concentrations of honey used in this study were based on other studies that utilise honey as intervention. Nordin et al. (2018) used freeze-dried kelulut honey ranging from 0.012 µg/ml to 25 600 µg/ml while Zivkovic et al. (2018) used manuka honey with concentrations of 25, 50, 100, 250, 500 and 1000 µg/ml. Low doses of honey was found to be more effective in exhibiting its properties as compared to high doses. A higher concentration of kelulut honey (1 mg/ml to 25 mg/ml) results in reduced cell viability (data not shown).

The relative percentage of LCL viability in this study increased significantly when treated with kelulut honey for 24 hours. The highest viability (164.64%) was observed when the cell was treated with 250 µg/ml of kelulut honey ($p < 0.01$). The viability then started to decline when treated with higher concentration of honey. Similar pattern has been observed in human fibroblast cells treated with kelulut honey as wound healing model (Nordin et al. 2018) and in murine fibrosarcoma cell line L929 when treated with gelam honey revealing its anti-inflammatory property (Kassim et al. 2010). This observation is caused by harbouring various phenolic compounds in honey such as hesperetin, ellagic acid, and quercetin (Kassim et al. 2010). At different concentrations of honey, different phenolic compound will be its prominent active component thus resulting in the increment of cell viability in a concentration-dependent manner, and a decline at a certain dosage.

We predicted that kelulut honey lowers the oxidative stress and cytotoxicity in LCL thus enhancing its viability. Gelam honey has been proposed to reduce oxidative damage through modulation of the activity of endogenous antioxidant enzymes, catalase and superoxide dismutase (Sahhugi et al. 2014). This antioxidant property of honey is attributed to its free radical scavenging activity of honey by its phenolics and flavonoid compounds (A-Rahaman et al. 2013; Abd Jalil et al. 2017; Adekoya et al. 2016; Afanas'ev et al. 1989; Beretta et

al. 2007; Cianciosi et al. 2018; Kishore et al. 2011). The free radical scavenging activity of honey was shown to suppress TNF- α -induced cytotoxicity caused by reactive oxygen species (ROS) (Delhalle et al. 2002; Kassim et al. 2010). Honey through its phenolics also able to provide protection against the lipid peroxidation thus reducing and preventing diseases (Cianciosi et al. 2018).

Our finding was similar to an earlier study which used endothelial cell culture. In the study, honey was able to reduce and eliminate ROS in endothelial cell induced with oxidative stress. In addition to that, the study showed that honey, through its phenolics and flavonoids protected endothelial cells exposed to peroxy radicals from 1,1-diphenyl-2-picrylhydrazyl (AAPH) and to hydrogen peroxide (Beretta et al. 2007).

There are a few limitations in this study. First, this study only uses one type of stingless bee honey to measure the viability of LCL whereas there are other stingless honey and sting bee honey. Second, this study does not identify the phenolics and flavonoid compound presence in the honey. Detailed identification of the phenolics and flavonoids of stingless bee honey have been determined using liquid chromatography-mass spectrometry by Ranneha et al. (2018) and Kharsa (2017) whereas the other properties of honey such as pH and ash content has been determined by (Fatima et al. 2018).

CONCLUSION

To the best of our knowledge, this work is the first report to determine the antioxidant activity of kelulut honey and its effect on the viability of LCL. Kelulut honey supplementation shows a significant effect towards increasing the viability of LCL. Kelulut honey is a potent antioxidant that has high total phenolic content, total flavonoid content and FRAP. Analysis of FRAP was found to have strong correlations with the total phenolic and flavonoid contents. This analysis showed that the phenolic and flavonoid contents in kelulut honey may be the active compound that contributes to oxidative damage protection of LCL thus enhancing the cell viability.

ACKNOWLEDGEMENT

This work was supported by a grant from the Fundamental Research Grant Scheme (FRGS/1/2016/SKK06/UKM/03/1). Methods for determination of TPC, TFC and FRAP were provided by Noor Baitee A Rahim.

REFERENCES

- A-Rahaman, N.L., Chua, L.S., Sarmidi, M.R., Aziz, R. 2013. Physicochemical and radical scavenging activities of honey samples from Malaysia. *Agricultural Sciences* 4: 46-51.
- Abd Jalil, M.A., Kasmuri, A.R., Hadi, H. 2017. Stingless bee honey, the natural wound healer: a review. *Skin Pharmacol Physiol* 30(2): 66-75.
- Abdullah, B., Lazim, N.M., Salim, R. 2015. The effectiveness of Tualang honey in reducing post-tonsillectomy pain. *Kulak Burun Bogaz Ihtis Derg* 25(3): 137-43.
- Adekoya, A.A., Ahmad, S., Maziah, M. 2016. Assessment of total phenolic compounds and in vitro free radical scavenging potentials of water extracts of ten selected species of Zingiberaceae rhizomes use in folkloric medicine. *Pak J Pharm Sci* 29(3): 979-84.
- Afanas'ev, I.B., Dorozhko, A.I., Brodskii, A.V., Kostyuk, V.A., Potapovitch, A.I. 1989. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem Pharmacol* 38(11): 1763-9.
- Al-Mamary, M., Al-Meeri, A., Al-Habori, M. 2002. Antioxidant activities and total phenolics of different types of honey. *Nutr Res* 22(9): 1041-7.
- Al-Rahbi, B., Zakaria, R., Othman, Z., Hassan, A., Mohd Ismail, Z.I., Muthuraju, S. 2014. Tualang honey supplement improves memory performance and hippocampal morphology in stressed ovariectomized rats. *Acta Histochem* 116(1): 79-88.
- Aziz, C.B., Ismail, C.A., Hussin, C.M., Mohamed, M. 2014. The antinociceptive effects of tualang honey in male sprague-dawley rats: a preliminary study. *J Tradit Complement Med* 4(4): 298-302.
- Baba, S.A., Malik, S.A. 2015. Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii* Blume. *J Taibah Univ Sci* 9(4): 449-54.
- Benzie, I.F., Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 239(1): 70-6.
- Beretta, G., Granata, P., Ferrero, M., Orioli, M., Maffei Facino, R. 2005. Standardization of antioxidant properties of honey by a combination of spectrophotometric/fluorimetric assays and chemometrics. *Anal Chim Acta* 533(2): 185-91.
- Beretta, G., Orioli, M., Facino, R. M. 2007. Antioxidant and radical scavenging activity of honey in endothelial cell cultures (EA.hy926). *Planta Med* 73(11): 1182-9.
- Bertoncelj, J., Doberšek, U., Jamnik, M., Golob, T. 2007. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. *Food Chem* 105(2): 822-8.
- Bogdanov, S., Jurendic, T., Sieber, R., Gallmann, P. 2008. Honey for nutrition and health: a review. *J Am Coll Nutr* 27(6): 677-89.
- Breen, M.S., White, C.H., Shekhtman, T., Lin, K., Looney, D., Woelk, C.H., Kelsoe, J.R. 2016. Lithium-responsive genes and gene networks in bipolar disorder patient-derived lymphoblastoid cell lines. *Pharmacogenomics J* 16(5): 446-53.
- Budin, S.B., Jubaidi, F.F., Mohd Noor Azam, S.N.F., Mohamed Yusof, N.L., Taib, I.S., Mohameda, J. 2017. Kelulut honey supplementation prevents sperm and testicular oxidative damage in streptozotocin-induced diabetic rats. *Diagnostic & Applied Health Sciences* 79(3): 89-95.
- Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C. 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 10(3): 178-82.

- Chua, L.S., Rahaman, N.L., Adnan, N.A., Eddie Tan, T.T. 2013. Antioxidant Activity of Three Honey Samples in relation with Their Biochemical Components. *J Anal Methods Chem* 2013: 313798.
- Cianciosi, D., Forbes-Hernández, Y.T., Afrin, S., Gasparrini, M., Reboledo-Rodríguez, P., Manna, P.P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., Quiles, L.J., Giampieri, F., Battino, M. 2018. Phenolic compounds in honey and their associated health benefits: a review. *Molecules* 23(9): pii: E2322.
- Delhalle, S., Derogowski, V., Benoit, V., Merville, M.P., Bours, V. 2002. NF-kappaB-dependent MnSOD expression protects adenocarcinoma cells from TNF-alpha-induced apoptosis. *Oncogene* 21(24): 3917-24.
- Erejuwa, O.O., Sulaiman, S.A., Ab Wahab, M.S. 2012. Honey: a novel antioxidant. *Molecules* 17(4): 4400-23.
- Fatima, I.J., Mohd Hilmi, A.B., Salwani, I., Lavaniya, M. 2018. Physicochemical characteristics of malaysian stingless bee honey from trigona species. *IMJM* 17: 187-91.
- Fereres, F., Andrade, P., Tomás-Barberán, F.A. 1994. Flavonoids from Portuguese heather honey. *Z Lebensm Unters Frosch* 199(1): 32-7.
- Gheldof, N., Wang, X., Engeseth, N. 2002. Identification and quantification of antioxidant components of honeys from various floral sources. *J Agric Food Chem* 50(21): 5870-7.
- Goon, J.A., Gunasekaran, G., Muhamad Fitri, C.A., Chandrashegkar, S., Hajar Amalnina, A.B., Raishan, S., Nurul Faiznani, Z., Ismail, N.A.S., Mohd Yusof, Y.A. 2017. Effects of gelam honey on oxidative stress in lung cancer cells. *Med Health* 12(2): 202-9.
- Hassan, H., Abdul Karim, N. 2018. Honey as versatile remedy: a focus on selected honeys. *Int J Food Nutr Sci* 6(1): 1-6.
- Hussain, T., Mulherkar, R. 2012. Lymphoblastoid Cell lines: a Continuous in Vitro Source of Cells to Study Carcinogen Sensitivity and DNA Repair. *Int J Mol Cell Med* 1(2): 75-87.
- Isla, M.I., Craig, A., Ordoñez, R., Zampini, C., Sayago, J., Bedascarrasbure, E., Alvarez, A., Salomón, V., Maldonado, L. 2011. Physicochemical and bioactive properties of honeys from Northwestern Argentina. *LWT Food Sci Technol* 44(9): 1922-30.
- Iurlina, M.O., Saiz, A.I., Fritz, R., Manrique, G.D. 2009. Major flavonoids of Argentinean honeys. Optimisation of the extraction method and analysis of their content in relationship to the geographical source of honeys. *Food Chem* 115(3): 1141-9.
- Jubri, Z., Rahim, N.B., Aan, G.J. 2013. Manuka honey protects middle-aged rats from oxidative damage. *Clinics* 68(11): 1446-54.
- Kassim, M., Achoui, M., Mustafa, M.R., Mohd, M.A., Yusoff, K.M. 2010. Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutr Res* 30(9): 650-9.
- Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W., Chua, L.S. 2014. Total phenolic contents and colour intensity of Malaysian honeys from the *Apis* spp. and *Trigona* spp. bees. *Agric Agric Sci Procedia* 2: 150-5.
- Kharsa, B.E. 2017. *Physiochemical and antioxidant properties of Malaysian Trigona and tualang bee honey*. (Master of Health Sciences (Nutrition Sciences)), International Islamic University Malaysia.
- Kishore, R.K., Halim, A.S., Syazana, M.S., Sirajudeen, K.N. 2011. Tualang honey has higher phenolic content and greater radical scavenging activity compared with other honey sources. *Nutr Res* 31(4): 322-5.
- LeBlanc, J.G., Chain, F., Martín, R., Bermúdez-Humarán, L.G., Courau, S., Langella, P. 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microb Cell Fact* 16(1): 79.
- Machado De-Melo, A.A., Almeida-Muradian, L. B.d., Sancho, M.T., Pascual-Maté, A. 2018. Composition and properties of *Apis mellifera* honey: A review. *J Apic Res* 57(1): 5-37.
- Martinotti, S., Bucekova, M., Majtan, J., Ranzato, E. 2018. Honey: an effective regenerative medicine product in wound management. *Curr Med Chem*.
- Martinotti, S., Ranzato, E. 2018. Honey, Wound Repair and Regenerative Medicine. *J Funct Biomater* 9(2): pii: E34.
- Mohan, A., Quek, S.-Y., Gutierrez-Maddox, N., Gao, Y., Shu, Q. 2017. Effect of honey in improving the gut microbial balance. *Food Qual Saf* 1(2): 107-15.
- Morrison, G., Lenkala, D., LaCroix, B., Ziliak, D., Abramson, V., Morrow, P. K., Forero, A., Van Poznak, C., Rugo, H.S., Nanda, R., O'Donnell, P.H., Huang, R.S. 2016. Utility of patient-derived lymphoblastoid cell lines as an ex vivo capecitabine sensitivity prediction model for breast cancer patients. *Oncotarget* 7(25): 38359-66.
- Niu, N., Schaid, D.J., Abo, R.P., Kalari, K., Fridley, B. L., Feng, Q., Jenkins, G., Batzler, A., Brisbin, A. G., Cunningham, J.M., Li, L., Sun, Z., Yang, P., Wang, L. 2012. Genetic association with overall survival of taxane-treated lung cancer patients - a genome-wide association study in human lymphoblastoid cell lines followed by a clinical association study. *BMC Cancer* 12: 422.
- Nordin, A., Omar, N., Sainik, N.Q.A.V., Chowdhury, S.R., Omar, E., Saim, A., Idrus, R. 2018. Low

- dose stingless bee honey increases viability of human dermal fibroblasts T that could potentially promote wound healing. *Wound Medicine* **23**: 22-27.
- Othman, Z., Shafin, N., Zakaria, R., Hussain, N. H., Mohammad, W.M. 2011. Improvement in immediate memory after 16 weeks of tualang honey (Agro Mas) supplement in healthy postmenopausal women. *Menopause* **18**(11): 1219-24.
- Pisoschi, A.M., Negulescu, G.P. 2011. Methods for total antioxidant activity determination: a review. *Biochem Anal Biochem* **1**(106): 1-10.
- Prior, R.L., Wu, X., Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* **53**(10): 4290-302.
- Putri Shuhaili, S., Haszianaliza, H., Muhammad Fairuz, A., Zar Chi, T. 2016. Gelam honey: a review of its antioxidant, anti-inflammatory, anticancer and wound healing aspects. *Med Health* **11**(2): 105-16.
- Ranneha, Y., Ali, F., Zareia, M., Md Akimb, A., Abd Hamid, H., Khazaai, H. 2018. Malaysian stingless bee and Tualang honeys: A comparative characterization of total antioxidant capacity and phenolic profile using liquid chromatography-mass spectrometry. *LWT - Food Science and Technology* **89**: 1-9.
- Sahhugi, Z., Hasenan, S.M., Jubri, Z. 2014. Protective effects of gelam honey against oxidative damage in young and aged rats. *Oxid Med Cell Longev* **2014**: 673628.
- Saiful Yazan, L., Muhamad Zali, M.F., Mohd Ali, R., Zainal, N.A., Esa, N., Sapuan, S., Ong, Y. S., Tor, Y. S., Gopalsamy, B., Voon, F. L., Syed Alwi, S.S. 2016. Chemopreventive properties and toxicity of Kelulut Honey in *Sprague Dawley* Rats induced with azoxymethane. *Biomed Res Int* **2016**: 4036926.
- Sawazaki, T., Nakajima, Y., Urai, T., Mukai, K., Ohta, M., Kato, I., Kawaguchi, A., Kinoshita, Y., Kumagai, Y., Sakashita, A., Yamazaki, A., Nakatani, T. 2018. Efficacy of Honeydew Honey and Blossom Honey on Full-thickness Wound Healing in Mice. *Wounds* **30**(7): 197-204.
- Sie, L., Loong, S., Tan, E.K. 2009. Utility of lymphoblastoid cell lines. *J Neurosci Res* **87**(9): 1953-9.
- Singleton, V.L., Orthofer, R., Lamuela-Raventós, R. M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol* **299**: 152-78.
- Stagos, D., Soulitsiotis, N., Tsadila, C., Papaconomou, S., Arvanitis, C., Ntontos, A., Karkanta, F., Adamou-Androulaki, S., Petrotos, K., Spandidos, D.A., Kouretas, D., Mossialos, D. 2018. Antibacterial and antioxidant activity of different types of honey derived from Mount Olympus in Greece. *Int J Mol Med* **42**(2): 726-34.
- Yazan, L.S., Zainal, N.A., Ali, R.M., Zali, M.F.S.M., Ong, Y.S., Tor, Y.S., Gopalsamy, B., Ling, V.F., Sapuan, S., Esa, N., Haron, A.S., Ansar, F.H.Z., Mokhtar, A.M.A. and Alwi, S.S.S, 2018. Antiulcer properties of kelulut honey against ethanol-induced gastric ulcer. *Pertanika J Sci Technol* **26**(1): 121-32.
- Zainol, M.I., Mohd Yusoff, K., Mohd Yusof, M. Y. 2013. Antibacterial activity of selected Malaysian honey. *BMC Complement Altern Med* **13**: 129.
- Zivkovic, L., Bajic, V., Dekanski, D., Cabarkapa-Pirkovic, A., Giampieri, F., Gasparrini, M., Mazzoni, L., Potparevic, B. S. 2018. Manuka honey attenuates oxidative damage induced by H₂O₂ in human whole blood in vitro. *Food Chem Toxicol* **119**: 61-5.

Received: 7 Aug 2018

Accepted: 8 Mar 2019