ORIGINAL ARTICLE

Quality Characteristics, Antioxidant and Anticancer Potential of Stingless Bee Honey and Honeybee Honey from Similar Environmental Conditions

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ABSTRACT

INTRODUCTION: The composition of honey depends on intrinsic and extrinsic factors, including place of origin, the floral types, the season, and the storage conditions. This study investigates the effect of bee species (Meliponini or Apis) on the quality characteristics, antioxidant, phytochemical, and anticancer potential of unprocessed stingless bee honey (SBH) and honeybee honey (HBH) collected from the same environmental factors including the location of the nest, floral type (Acacia), harvesting month (August) and storage conditions (<5°C). **MATERIALS AND METHODS**: To measure the honey quality, 5-hydroxymethylfurfural (HMF) and diastase activity were analysed, followed by total phenolic content (TPC) and total flavonoid content (TFC) for phytochemicals. Antioxidant activity was assessed via a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Cytotoxicity towards cervical cancer cell line (HeLa) was measured using a 2-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (IC50). **RESULTS**: The results indicated that HBH exhibited significantly higher diastase activity (2.17 ± 0.07 DN), TPC (338.95 ± 3.49 mg GAE/kg); better DPPH scavenging activity EC50 (80.06 mg/ml) and MTT activity IC50 (64.80 mg/ml) towards HeLa as compared to SBH with absence of diastase activity, TPC (250.60 ± 3.98 mg GAE/kg), DPPH scavenging activity EC50 (165.80 mg/ml) and MTT activity IC50 (75.76 mg/ml). However, there were no significant differences in HMF and TFC between HBH and SBH. **CONCLUSION**: Even though many believe that SBH is superior to HBH, the present study found that in similar environmental conditions, HBH demonstrates better antioxidant capacity, anticancer potential, and phenolic content as compared to SBH.

Keywords
Stingless bee honey, honeybee honey, anticancer potential, honey quality, antioxidant activity.

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INTRODUCTION

Honey is mainly made up of sugars and other minor substances including proteins, enzymes, amino acids, organic acids, vitamins, polyphenols, minerals, and phytochemicals such as flavonoids and carotenoids. These compounds are associated with the therapeutic benefits of honey.[1] Previous studies suggested that honey contains ingredients that offer antioxidant, antimicrobial, anti-inflammatory, and anticancer properties with broad positive effects against many diseases.[2] Research has proven the ability of honey to prevent many diseases.[3]

Honey from the *Apis* species is the most common type of honey and is widely used around the globe. *Apis* is native to Europe, western Asia, and Africa.[4] In South-East Asia, bee honey is mainly used in health care and is commonly described as the best producer of honey, as it is adaptable to environmental changes, especially *A. mellifera*.[5] The stingless bee is a small type of bee which is known as *Meliponini sp* or *Trigona sp.* This bee is also known as *kelulut* in Malaysia. Stingless bees are associated with low honey productivity, with only 4 kg per colony annually. Honeybee honey and stingless bees have been reported to differ in terms of morphology, nectar collection, foraging activity, and hive build.[6]

Honey's physicochemical composition is crucial in the determination of honey quality. Hydroxymethylfurfural (HMF), for instance, is an indicator of the deterioration of

honey and is formed during the decomposition of monosaccharides via Maillard's reaction. Meanwhile, diastase activity is an important determinant of honey quality. Extensive heating and high acidity result in a lower diastase number (DN) due to the denaturation of the enzyme.[7] The phenolic acids of honey include gallic, coumaric, and caffeic acids, and flavonoids such as catechin, kaempferol, and apigenin.[8] These compounds are the main contributor to the potency of honey as an antioxidant. Reactive oxygen species (ROS) are the causer of oxidative stresses that lead to various diseases and disorders such as cardiovascular disease and cancer.[9] Several studies have claimed that both honey bee honey (HBH) and stingless bee honey (SBH) are very effective in combating ROS and are one of the best antioxidant sources that can be acquired naturally.[10,11]

A thorough understanding of the characteristics of honey is also required for commercialization. However, studies involving samples that are taken from different geographical sources may produce variations in results as the compositions of honey differ according to their floral, geographical, and entomological sources, and are also influenced by extrinsic factors such as the environment, seasons, storage conditions, time and processing.[12] Therefore, a comparative study of samples obtained from an identical geographical source and environment is required to identify the difference in characteristics between the different types of honey. There were studies reported on the difference in honey composition affected by different bee species.[6,13] The physicochemical properties, sensory characteristics, and glycemic load of SBH and HBH have been reported previously, with a significant difference in moisture content observed between the different types of honey obtained from the same geographical source, but no significant difference in ash content, pH and electrical conductivity observed. In addition, raw and unprocessed HBH was much more preferred as compared to SBH based on sensory evaluation with both of the samples categorised as low glycemic load (GL) that are suitable to be consumed by diabetic patients with moderate intake.[13]

Although various studies have been conducted previously on SBH and HBH, further studies are still required to obtain a clear distinction between different types of honey by obtaining samples from the same geographical source and providing further information for its commercialization. Therefore, the present study sought to compare the cytotoxicity, quality, and antioxidant activity of SBH and HBH honey from the same location (Marang, Malaysia), floral type (Acacia), and harvest time (June to August).

MATERIALS AND METHODS

Sample preparation

SBH and HBH were obtained from the Big BEE honey farm in Marang, Terengganu. The honey was stored at a temperature ranging from 1-5°C and then left at room temperature for about 2 hours prior to any assays.

Determination of hydromethylfurfural (HMF) content

The HMF content of honey was determined using a method proposed by the International Honey Commission (IHC).[14] The HMF (mg/kg) was calculated using the following formula:

$$HMF\left(\frac{mg}{100mg}\right) = \frac{(A284 - A336) \times 74.87}{weight\ honey}$$

Measurement of diastase number (DN)

The diastase number (DN) of the honey was determined using the Phadebas® Honey Diastase Test kit (Pharmacia Diagnostics, Sweden).[14] Analysis was performed according to the manufacturer's instructions. The blank was prepared with the same treatment by using acetate buffer without a sample.

Total phenolic content (TPC) and total flavonoids content (TFC)

Total phenolic content (TPC) was measured based on the method used by Ibrahim Khalil *et al.* [15] The absorbance was recorded at wavelength 765 nm and TPC was expressed as mg GAE/kg via the calibration of gallic acid.

Total flavonoid content (TFC) was measured by mixing 1.0 ml of diluted honey with 4.0 ml of distilled water, 0.3 ml sodium nitrite, 0.3 ml aluminium chloride, and 0.3 ml NaOH. The absorbance of the solution was then recorded at 510 nm and TFC was expressed as mg CEQ/kg.[15]

DPPH scavenging activity

The DPPH assay was done as reported previously.[16] A volume of 20 µl of honey samples at various concentrations were loaded into 96-well plates, along with 200 µl of DPPH solution (2.37 µg/ml in methanol). The result was reported at a half-maximal effective concentration (EC_{50}).

Cytotoxicity assay

A cervical cancer cell line (HeLa) was purchased from ATCC (Virginia, USA). Cells were maintained in MEM medium

supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% of non-essential amino acid, 1% of sodium pyruvate and incubated in a humidified incubator with 5% CO_2 at 37°C according to ATCC® HTB-22™. The honey was filtered by using a sterile nylon syringe filter of 0.45 micron and a stock solution of 500 mg/ml in DMSO was prepared.

A cell cytotoxicity assay was performed based on the previously described procedure.[17] Cells (8 x 10^4/well) were harvested and seeded on 96-well plates for 24 h. The cells were then treated with honey at different concentrations and further incubated at 37°C, 5% CO_2 for 72 h. Cell inhibition was expressed in terms of inhibitory concentration (IC_{50}), which refers to the concentration of honey needed to inhibit half of the maximum cancer cell population.

Cell morphology

Cells (8 x 10^4 cells/ml) were seeded into a 96-well plate and incubated overnight at 37°C with 5% CO_2 and 95% water in a humidified incubator. Then, the desired concentrations of samples were loaded into the plate. The plate was incubated for 72 hours. Morphological changes in the cells were observed and captured under an inverted microscope (Olympus IX50, Pennsylvania, USA).

Statistical analysis

The triplicate data obtained were presented as mean ± standard deviation. Independent t-test and one-way ANOVA with post-hoc Tukey's test were performed using Statistical Package for Social Science (SPSS) version 16 software. In all analyses, $p < 0.05$ indicates a significant difference.

RESULTS

5- Hydroxymethylfurfural (HMF)

The comparison of the quality and phytochemical content of the honey samples is presented in Table I. The quality of honey was measured based on HMF content whereby no differences in HMF content were observed from both SBH and HBH (<5 mg/kg).

Table I: Comparison of honey quality and phytochemical content.

	HMF (mg/kg)	Diastase (DN)	TPC (mg GAE/kg)	TFC (mg CEQ/kg)
SBH	<5.00	0.0^a	250.60 ± 3.98^a	46.85 ± 3.72^a
HBH	<5.00	2.17 ± 0.073^b	338.95 ± 3.49^b	38.95 ± 3.72^a

Results are mean of triplicate with standard deviation. Letter (^{a} and ^{b}) indicate a significant difference ($p < 0.05$) between kinds of honey by Independent t-test.

Diastase activity

Diastase activity was also measured to determine the quality of the honey samples (Table I). The present study found that diastase activity was not detected in SBH, while HBH showed a small amount of diastase activity at 2.168 DN, as shown in Table I. This value was lower than IHC standards, as high-quality honey should at least contain 8 DN.

Total phenolic content (TPC) and total flavonoid content (TFC)

In terms of TPC content, SBH with 250.60 ± 3.98 mg GAE/kg yielded a significantly lower TPC as compared to HBH with 338.95±3.49 mg GAE/kg as shown in Table I ($p < 0.05$). Furthermore, the total flavonoid content (TFC) of both HBH and SBH samples was not statistically different ($p > 0.05$), with raw Acacia honeybee honey yielding 38.95 3.72 mg/kg of TFC and SBH yielding 46.85 3.72 mg/kg of TFC.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Table II shows DPPH radical inhibition of HBH, SBH, and quercetin. From the results obtained, significantly different scavenging activity was observed between samples at all concentrations. Both HBH and SBH exhibited strong DDPH inhibition at concentrations of 250.0 mg/ml, with higher than 50% inhibition. Quercetin as the positive control exhibits stronger DPPH radical inhibition as compared to both of the honey samples with significantly higher inhibition at lower concentrations of 1.95 mg/ml. There was no inhibition reaction observed for SBH at a concentration lower than 3.91 mg/ml, while HBH showed no reaction at 1.95 mg/ml of sample concentration

Table II: DPPH inhibition in percentage with various concentrations of honeybee honey, stingless bee honey, and positive control (quercetin).

Concentration	DPPH Inhibition %		
(mg/ml)	HBH	SBH	Quercetin
250.00	66.00 ± 1.41^b	57.90 ± 2.97^c	100.00^a
125.00	63.92 ± 0.12^b	40.84 ± 0.23^c	100.00^a
62.50	40.17 ± 0.23^b	25.89 ± 0.16^c	89.89 ± 14.30^a
31.25	23.20 ± 0.18^b	3.83 ± 0.23^c	81.50 ± 0.71^a
15.63	9.83 ± 0.24^b	1.83 ± 0.23^c	81.39 ± 0.87^a
7.83	7.67 ± 0.47^b	1.04 ± 0.05^c	82.75 ± 0.35^a
3.91	5.17 ± 0.24^b	0^c	80.84 ± 1.18^a
1.95	0^b	0^b	58.170 ± 0.23^a

Results are the mean and standard deviation of duplicate. In the same row, different letters (^{a}, ^{b}, and ^{c}) indicate significant differences ($p<0.05$) by one-way ANOVA.

Notes: SBH (Raw stingless bee honey) and HBH (Raw honeybee honey)

Figure 1 shows the effective concentration (EC_{50}) of samples with quercetin as the positive control. The results show that the EC_{50} of honeybee honey was significantly lower than stingless bee honey. This indicates that honeybee honey is a better free radical scavenger at lower concentrations as compared to stingless bee honey.

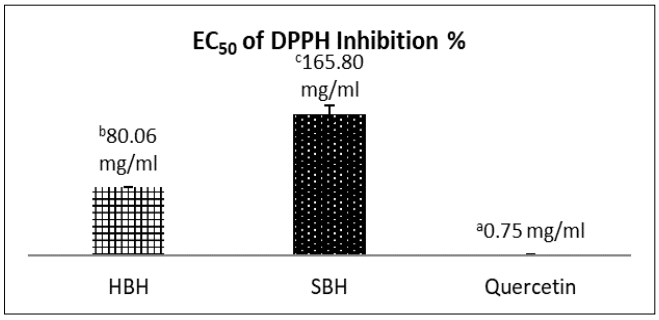

Figure 1: The effective concentration (EC_{50}) of honey towards DPPH radicals. Different letters ([a], [b], and [c]) indicate significant differences ($p<0.05$) by one-way ANOVA. Quercetin as a positive control.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

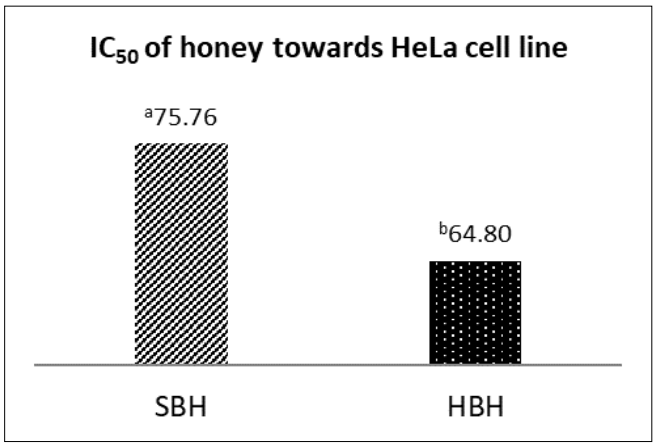

Figure 2: The inhibitory concentration of honey (mg/ml) towards cervical cancer (HeLa) cell lines. Different letters ([a] and [b]) indicate significant differences ($p<0.05$) by the Independent t-test.

Cytotoxicity was expressed in terms of inhibitory concentration (IC_{50}), defined as the concentration of honey required to kill 50% of cells, as shown in Figure 2. A higher IC_{50} value towards the HeLa cell line was observed for Stingless bee honey as compared to honeybee honey samples with a significant difference ($p<0.05$) observed between samples. This indicated that honeybee honey offered a higher anticancer potential as compared to stingless bee honey with a lower sample concentration required for inhibition. Moreover, both SBH and HBH are very cytotoxic towards the HeLa cell line at concentrations higher than 31.25 mg/ml, with a 27.38% and 29.94% decrease in cell viability respectively. The IC_{50} value of HBH was significantly lower than SBH, indicating that HBH exhibited higher toxicity towards HeLa.

The morphology of HeLa cells was observed after 72h of exposure to honey at different concentrations. From Figure 3, cells treated with both kinds of honey showed morphological changes, with the presence of clumping of dead cells.

DISCUSSION

Table 1 shows that the HMF values of the honey samples were below 5 mg/kg, indicating that both of these kinds of honey were fresh and high in quality.[18] Low HMF values were also recorded in other studies involving raw honey. For example, no HMF was detected in eight types of honey,[18] 0.0 mg/kg – 11.45 mg/kg in 45 types of honey,[19] and 0.0 mg/kg – 1.6 mg/kg from 46 types of honey.[20] Low HMF indicated freshness and high-quality honey.[18] On the other hand, high HMF content is associated with deterioration, heat exposure, poor handling, and inadequate storage conditions.[21,22] The maximum value of HMF set by the Codex Alimentarius Commission[23] for honeybees originated from non-tropical regions is 40 mg/kg, while the Malaysian Standard for honey has established a value lower than 30 mg/kg as permissible.[24]

Low diastase activities were also detected in several species of Malaysian SBH from Kelantan ranging from 1.22-1.97 DN.[25] According to Malaysian standards, no minimum value was set for the diastase activity of Malaysian honey. Diastase can be

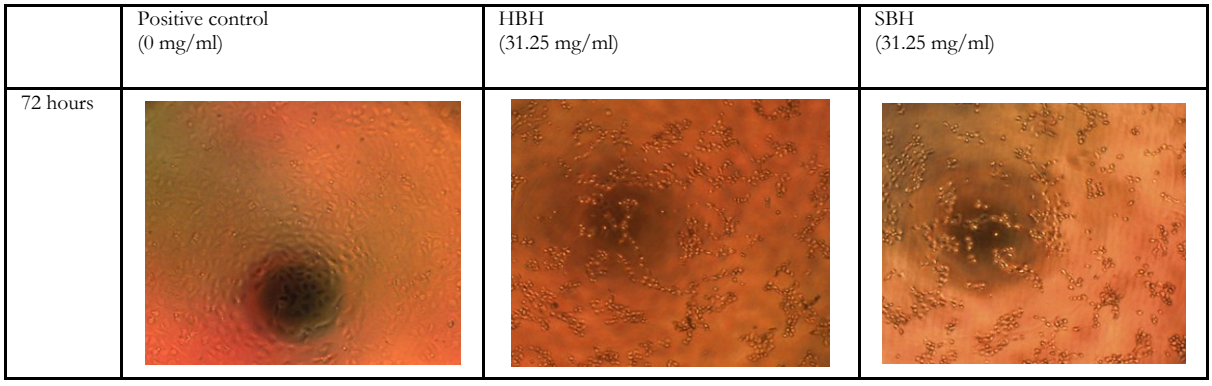

Figure 3: Cell morphology of HeLa at 72 hours of treatment with 31.25 mg/ml of honey at a magnification of 40X.

reduced over time due to denaturing when exposed to heat and low pH. Malaysian honey is known to have high moisture levels due to its tropical climate which caused an increase in the fermentation of yeasts that releases acid as a by-product, potentially decreasing the pH. This further leads to a shorter half-life of the enzyme, rendering it difficult to be used as a quality parameter.[24] Apart from quality, diastase activity can also be utilized for the determination of honey purity, as the enzyme is involved in sugar conversion and is only present in the stomachs and saliva of bees.[25]

The results obtained for the total phenolic content (TPC) of honey are in contrast with a previous study by Peng *et al.*[26], which reported that SBH yielded up to 33% higher TPC as compared to HBH. However, unlike the present study, the origin, floral sources, or season of samples were not stated in the previous study.[26] The TPC of honey has been reported to be dependent on floral sources, botanical origins, season, environment, and pigment, which may contribute to differences in the results.[12] In the present study, all of these attributes including the time of harvest and place of the samples tested were identical which indicated that these differences may be contributed by other factors. It is postulated that the bee species are responsible for these differences. A study found significant differences in most of the honey composition in 23 species of stingless bees.[27] Besides, another study also indicated the different compositions of honey produced by different species of bees who shared a similar botanical origin.[28] However, both studies did not clearly explain the factor that contributes to the different composition of honey from different bee species. The possible reason is may be due to the process of honey production by the bees and also the storage of the honey. Stingless bee kept their honey in ellipsoidal pots made with cerumen. Unlike honey bee honey, the high moisture in the cerumen pots of stingless bees allows the biological transformation to occur through the fermentation process by yeasts and bacteria in the pots.[29] As the phytochemical composition of honey is closely related to the presence of the phytochemicals in the cerumen,[30] these may explain the difference of the honey bee honey quality compared to the stingless bee. Stadelmeier and Bergner[31] also found the difference in the enzyme activity of two different bee species which may be related to the difference in honey production.

In addition, the present study also found that the TPC of HBH is higher than the previously reported value by Krpan *et al.*[32] with 43.66 ± 6.45 GAE mg/kg. Another study on three samples of Acacia honeybee honey reported a range of between 249 GAE mg/kg-323 GAE mg/kg.[33] Raw stingless Acacia honey has a significantly lower total phenolic content than raw honeybee honey ($p<0.05$) with 250.60 ± 3.980 GAE mg/kg. Furthermore, the TPC for SBH was found to be lower than the results of a study conducted on two types of Acacia SBH from Johor and from Terengganu which recorded 558.60 ± 24.00 GAE mg/kg and 331.20 GAE mg/kg ± 9.10 of TPC respectively.[34] It has also been reported that the total phenolic content of Malaysian stingless bee honey from Perak ranges between 228.00 GAE mg/kg – 1058 GAE mg/kg.[35]

Phenolic acids have been widely studied and associated with reducing cardiovascular diseases and type-2 diabetes. Previous studies reported that honey contains various phenolic acids including gallic, syringic, benzoic, cinnamic, coumaric, and caffeic acids.[36] It also contributes to the anticancer properties of honey. A current review by Waheed *et al.*[1] reported that the phenolic acids in honey are the most crucial substance that provides anticancer activity properties and acts as a potent antioxidant.

In terms of the total flavonoid content (TFC) of honey, the TFC values of Acacia HBH from Malaysia ranges between 28.83– 113.06 mg/kg when harvested during all seasons.[37] These results are higher as compared to the TFC of acacia HBH from other countries such as Romania (9.1– 24.2 mg/kg), Italy (4.5– 10.1 mg/kg), and Burkina Faso (17.0– 83.5 mg/kg).[38] Meanwhile, four Malaysian SBHs from Gertak Sanggul, Jasin, Kubang Pasu, and Padang Terap showed lower TFC than the present study at 10.18 ± 1.04 mg/kg, 11.77 ± 0.80 mg/kg, 12.68 ± 0.14 mg/kg, and 10.27 ± 0.29 mg/kg, respectively.[39] On the other hand, it has been reported that two types of raw Malaysian stingless bee honey from Ayer Molek have flavonoid content of 82.38 ± 4.12 mg/kg and 60.95 ± 4.12 mg/kg of flavonoid content,[12] which is higher than the present study. From this data, it can be concluded that the flavonoid contents of honey are dependent on the places of origin.

The DPPH assay was developed by utilising free radicals to assess the antioxidant activity of a compound. It measures the scavenging capacity of antioxidants, with the reduction of odd electron nitrogen in DPPH by acquiring hydrogen atoms from antioxidants to the corresponding hydrazine.[40] According to one study, the EC_{50} of HBH ranged from 44.64 - 407.01 mg/ml, [32] which is consistent with the current study. Another author also reported that the EC_{50} value of Acacia honeybee honey was 29.85 mg/ml, and much more effective than the present study. Tualang and Gelam honey also reported good EC_{50} values, at 48.90 mg/ml and 15.70 mg/ml, respectively. Gelam honey exhibited a great free radical scavenging activity at a much lower concentration,[41] and a study on twenty-one kinds of honey from New Zealand reported EC_{50} values ranging from 7.5– 109 mg/ml.[42]

Meanwhile, SBH demonstrated less radical scavenging ability than the values reported in the previous study[34] where EC_{50} values of 97.24 mg/ml and 76.27 mg/ml for DPPH radicals were previously recorded. They also found that the EC_{50} values of stingless bee honey from starfruit and Gelam honey were 105.53 mg/ml and 32.58 mg/ml, respectively. A previous study reported that the EC_{50} values towards DPPH free-radical ranged from 52.33 mg/ml to 97.30 mg/ml in various places. The high radical scavenging activity of honey was mainly contributed by its phenolic and flavonoid contents, as stated previously in several studies.[12]

The cell cytotoxicity of honey was assessed by MTT assay, a sensitive and reliable indicator of cellular metabolic activity which is preferable over many other methods. The cytotoxicity of honey is due to its actions on cancer cells, including apoptosis induction and cell cycle arrest.[43] Honey with hydrogen peroxide (H_2O_2) accumulated as free radicals lead to oxidative stress in cancer cells, proceeded by lipid-peroxide activity that leads to cell death.

A study on Tualang honey with oral squamous cell carcinomas (OSCC) and human osteosarcoma (HOS) reported that the viability of both cells was reduced to as low as 15% with the presence of honey samples.[44] However, in the present study, lower concentrations of honey promoted cell viability up to 199.5% and 152.3%, respectively. These results were supported by a previous study whereby an increase in malignant glioma cancer cell proliferation was observed at a low concentration of honey but a high cytotoxic effect was observed at a higher concentration of greater than 5%.[45] The increase in cell viability indicated that honey promoted cervical cancer cell proliferation at lower concentrations. It is believed that glucose in honey may be the factor for the increase in cell viability and variation of the results. Glucose is a preferred nutrient for cancer cells, and sugars present in honey have been suggested to have both mutagenic, as well as antimutagenic effects. Honey is mainly composed of carbohydrates, with the major sugars in the form of fructose and glucose.[46]

The variability of the results is due to the difference in the cell used and the composition of the honey itself. Tualang honey induces apoptosis in MCF-7 and MDA-MB-231 cell lines by promoting depolarization of mitochondrial membrane that leads to the activation of the initiator caspases-8 and -9 which was followed by the activation of executioner caspases-3 and -7.[47] Manuka honey induced apoptosis in MCF-7 cells via the activation of caspases-9 and 6.[48] Based on Figure 2, low concentrations of honey promote HeLa proliferation up to 199.5%. The over-confluence cells can be seen on both of the honey at a concentration of 0.098 mg/ml. Glucose from sugars is believed to fuel developing cancer cells. Studies have shown that glucose promotes proliferation in endometrial cancer cell lines.[49] Another study on HPDE-6 cell line also found that glucose also promotes cell proliferation while increasing their oxidative stress.[50]

CONCLUSION

HBH demonstrates better phenolic content, antioxidant capacity and cytotoxicity towards cervical cancer cell line (HeLa) than SBH harvested from the same place, floral origin, and season. There were no significant differences between both HMF and flavonoid contents in both kinds of honey. All these kinds of honey have demonstrated the ability to inhibit HeLa growth at lower concentrations and exhibited high potential for use as an alternative chemotherapy drug. Both kinds of honey also exhibit antioxidant capacity and can be used in antioxidant supplements.

CONFLICT OF INTEREST

The authors declare no conflicts of interest. Financial support was obtained from the Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu, Malaysia.

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