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ORIGINAL ARTICLE

Cytotoxic Evaluation of Malaysian Kelulut Honey on Human Gingival Fibroblast Cell Line using MTT Assay

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ABSTRACT

Kelulut honey or stingless bee honey is a type of honey produced by stingless bees of the Trigona species where the nest is found in living trees. **Objective:** The aim of this study was to evaluate the cytotoxic potential of Malaysian Kelulut honey by employing MTT assay on a human gingival fibroblast cell line. **Methods:** Human gingival fibroblast cell line was cultured in minimal essential medium alpha (α-MEM) with 10% foetal bovine serum and 1% penicillin-streptomycin solution in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The cells were seeded at a cell density of $5x10^3$ cells/well in a 96-well culture plate for 24 hours. The cells were treated with seven different concentrations (200, 100, 50, 25, 12.5, 6.25, and 3.125mg/ml) of Malaysian Kelulut honey and incubated in a CO_2 incubator. The negative control comprised cells treated with growth media alone. The cell viability was assessed using MTT assay at 24, 48, and 72 hours. The test plate was shaken using a microplate shaker and the absorbance of the solution was measured at 570nm using an ELISA reader with the Magellan software. Statistical analysis of the data was carried out using Kruskal-Wallis test and SPSS 24.0.0 for Windows. A p value <0.05 was considered as statistically significant. **Results:** There was no cytotoxic effect of Malaysian Kelulut honey on HGF-1 based on the MTT assay at different concentrations and at different time points tested as the cell viability was above 70%. The highest percentage of cell viability at all three different durations of treatment were observed at 3.125mg/ml, whereas the lowest cell viability was observed at 200mg/ml of Kelulut honey concentration. However, statistically significant differences were seen between some of the concentrations at various time points. **Conclusion:** Since the cell viability of HGF-1 treated with Malaysian Kelulut honey was more than 70% at all concentrations ranging from 3.125mg/ml to 200mg/ml at three different time points (24, 48 and 72 hours), Malaysian Kelulut honey can be considered as non-cytotoxic on human gingival fibroblasts based on MTT assay under the present test conditions.

Key words: cytotoxicity, human gingival fibroblasts, Kelulut honey, MTT assay

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INTRODUCTION

Kelulut honey or stingless bee honey is a type of honey produced by stingless bees of the Trigona species where the nest is found in living trees.[1] Kelulut honey is characterised by its significant amount of flavour and fragrant qualities. It consists of high sugar content i.e., glucose, fructose, sucrose and maltose, as well as non-aromatic organic acids such as D-gluconic acid, malic acid and citric acid. It also consists of large amounts of bioactive substances, namely flavonoids, glucose oxidase catalase, and phenolic acids.

Kelulut honey has been perceived as a therapeutic honey as it exhibits high antioxidant,[1] antimicrobial,[2] and wound healing properties.[3] It is necessary to assess the cytotoxic nature of natural products as cytotoxicity is the characteristic of certain substances to be harmful to cell life. Cytotoxicity testing enables researchers to observe the growth, morphological effects, reproduction and proliferation of cells on treatment with substances having an unknown cytotoxic state.[4] This test also offers many ways of

detecting cell damage like monitoring morphological changes, cell growth and measurement of metabolic properties.[5-7] MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is one of the cytotoxic assays widely used in assessing cell viability and proliferation assay.[8-11] Human gingival fibroblasts (HGFs) are important cellular components involved in periodontal tissue repair.

Due to the dearth of information on the cytotoxicity of Kelulut honey on human gingival fibroblasts, this preliminary study aimed to assess the cell viability of human gingival fibroblasts treated with different concentrations of Malaysian Kelulut honey at different time periods using MTT assay. Moreover, it is of utmost importance to thoroughly investigate its benefits so that Malaysian stingless bee honey could contribute to the health of humans through its potential proliferative action.

METHODS

Cell line and culture

Human gingival fibroblast cell line (HGF-1) obtained from ATCC®CRL-2014™ (USA) was employed in the current research. HGF-1 were cultured until confluence in complete media comprising minimal essential medium alpha (α-MEM) (Gibco, Life Technologies, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, Life Technologies, USA), 1% penicillin (10000 units/ml) - streptomycin (10000 µg/ml) (Gibco, Life Technologies, USA) in a 5% CO_2 incubator (Nuaire, USA) at 37°C in a humidified atmosphere. The original medium was discarded, and the cells were washed using a phosphate buffered saline (PBS) (Gibco, Life Technologies, USA) before trypsinization using one ml of TryplETM Express stable trypsin (Gibco, Life Technologies, USA) in a 25cm^2 flask (SPL, USA) for the passaging process. Cells were left for three to four minutes in an incubator for the dissociation process and then the cells were neutralised using complete media (one ml for 25cm^2 flask). The suspensions were spun at 1200rpm for five minutes, and media and trypsin were discarded. Then, 1000µl of fresh complete medium was added to the cells and dispensed at 1:3 subculture ratio. HGF-1 were sub-cultured in three flasks (25cm^2) with four ml of culture medium. The cells were allowed to grow until it reached confluence and then the process of detaching the cells was repeated. The cells were seeded at a cell density of 5 x 103 cells/well in a 96-well culture plate (NuncTM, Denmark) for 24 hours for cell attachment at 37°C, 5% CO_2 and 95% relative humidity.

Malaysian kelulut honey sample

The Kelulut honey used in the current study was the one commercially marketed by Syamille Agrofarm & Resort Sdn. Bhd. (Kuala Kangsar, Perak, Malaysia).[12] This honey was obtained from the nests of stingless bees namely the Trigona species. Stingless bees after producing honey store it in resin pots in their hives.[13] For commercial use, honey is collected from the pot using suction pump and subsequently filtered to remove the impurities.[14]

MTT assay

After 24 hours of incubation, the extraction medium was filtered through a 0.22µm Millipore membrane filter (TPP, Switzerland). The extraction medium was serially diluted (200, 100, 50, 25, 12.5, 6.25, 3.125 mg/ml) in seven different 15 ml centrifuge tubes (Eppendorf, Germany). The diluted extracts were added into three 96 well-plates containing 5 x 103 HGF-1/well. The negative control comprised of cells treated with growth media alone. The 96 well plates containing the cells were then incubated in a 5% CO^2 incubator for 24, 48, and 72 hours at 37°C. Then, 10µl of MTT (Invitrogen, USA) with a concentration of 5 mg/ml was added to the culture medium and the plates were further incubated for four hours in a 5% CO^2 incubator at 37°C to enable the dye to be taken up by the remaining surviving cells. Total removal of the media containing the MTT solution was done and then cells were lysed with 100µl of dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) solution. The test plate was shaken using a microplate shaker for several minutes to solubilise the formazan crystals and the absorbance of the solution was measured at 570nm using an ELISA reader (Sunrise, TECAN, Austria) with the Magellan software. Three independent experiments were carried out with three replicates.

The cell viability was calculated using the following formula.

% viable cells = [A]test/[A]control x 100

Where A = absorbance

Data analysis

Data were analysed using SPSS Statistics for Windows, Version 24.0. and pairwise comparisons were done using the Kruskal-Wallis test. Statistical significance was set at $p<0.05$.

RESULTS

Cell viability of HGF-1 treated with different concentrations of Malaysian Kelulut honey

Cell viability of HGF-1 treated with Kelulut Honey at seven different concentrations (200, 100, 50, 25, 12.5, 6.25, and 3.125mg/ml) according to ISO 10993-12,[15] and negative control (cells treated with growth media only) at three different time periods (24, 48 and 72 hours) using MTT assay are shown in Table 1. The highest percentage of cell viability at all three different durations of treatment was observed at 3.125 mg/ml, whereas the lowest cell viability was observed at 200 mg/ml of Kelulut honey concentration. Pairwise comparisons of cell viability of HGF-1 treated with

Table 1. Cell viability of human gingival fibroblasts (HGF-1) treated with Malaysian Kelulut honey using MTT assay

Concentration (mg/ml)	Cell viability (%) Mean (SD)		
	24 hours	48 hours	72 hours
200	75.87 (4.14)	81.17 (6.18)	77.47 (3.61)
100	85.37 (2.02)	83.53 (5.11)	81.30 (0.96)
50	88.30 (2.55)	93.87 (6.99)	85.63 (2.99)
25	93.77 (1.23)	102.87 (5.35)	88.97 (3.19)
12.5	98.93 (1.45)	105.57 (7.37)	95.37 (3.74)
6.25	105.83 (6.91)	108.13 (8.54)	98.33 (3.45)
3.125	113.30 (7.31)	112.87 (6.40)	117.13 (6.43)
Control	100.00 (0.00)	100.00 (0.00)	100.00 (0.00)

Table 2. Pairwise comparison of cell viability of human gingival fibroblasts (HGF-1) treated with Malaysian Kelulut honey for 24, 48 and 72 hours using MTT assay

Variables	Median (IQR)	Chi square statistic (df)	p-value	Duration of incubation (hours)
Concentrations	4.50 (4.50)	42.567 (7)	0.000	24
Values	0.22 (0.05)			
Concentrations	4.50 (4.50)	16.956 (7)	0.018	48
Values	0.23 (0.06)			
Concentrations	4.50 (4.50)	37.434 (7)	0.000	72
Values	0.22 (0.04)			

Kruskal-Wallis test was performed as normality assumption was not met. The level of significance was set at 0.05 ($p<0.05$). Pairwise comparison: 200-25, 200-12.5, 200-control, 200-6.25, 200-3.125, 100-6.25, 100-3.125, 50-3.125 mg/ml are statistically significant ($p<0.05$), others are not significant at 24 hours of incubation. Pairwise comparison: 200-3.125 mg/ml is statistically significant ($p<0.05$), others are not significant at 48 hours of incubation. Pairwise comparison: 200-12.5, 200-6.25, 200-3.125, 100-3.125 mg/ml are statistically significant ($p<0.05$), others are not significant at 72 hours of incubation.

Malaysian Kelulut honey at three different time periods using MTT assay are presented in Table 2. However, statistical significance ($p<0.05$) was observed for the cell viability between the concentrations at 24, 48, and 72 hours (Table 2).

DISCUSSION

The Kelulut Honey used in this study was a commercial one which is rich in flavonoids, furfural and terpenoid. There are limited publications available on Malaysian stingless bee honey.[16] Most of the publications reported are on the physicochemical and antioxidant properties. The physicochemical properties of Kelulut honey that have been previously reported are moisture content (33.24 ± 2.54g/100 g), water activity (0.76 ± 0.03), specific gravity (1.36 ± 0.04), viscosity (0.29 ± 0.18 Pa.s), pH (3.26 ± 0.15), free acidity (136.8 ± 7.6 meq/kg), electrical conductivity (1.08 ± 0.37mS/cm) and colour intensity (990.3 ± 380.0mAU).[16] It has also been mentioned that the high water activity of Kelulut honey has a tendency of fermentation from bacteria and yeasts.[17] The low pH of Kelulut honey contributed to a sourly taste and its high electrical conductivity is related to the high ash (mineral) content.[16] The same authors also reported higher reducing power and also stronger antioxidant activity of Kelulut honey based on their studies. With regard to the nutritional composition, Kelulut honey showed high content of carbohydrate ranging from 67.20±0.11 to 73.01±0.35 g/100 g, potassium (701.33±26.27mg/kg), calcium (292.67±1.17mg/kg), magnesium (51.61±0.08mg/kg) and zinc (5.33±0.36mg/kg), while the phytochemical analysis showed that the total flavonoids and phenolic compounds ranged from 53.81±4.12 to 549.05±9.74 mg rutin/kg and 357.14±3.57 to 520.83±4.49mg gallic acid/kg respectively depending on the various solvents used for extraction.[18]

However, there is still a dearth of information on the cytotoxicity of Malaysian Kelulut honey on the normal cell line. Human gingival fibroblasts (HGFs) are the most abundant cell types in the periodontal connective tissues that function in repairing periodontal tissues and play a role in inflammatory periodontal diseases.[19] Supraja et al. described that HGFs are one of the common cell types that are used in cell cultures as they are easy to culture following enzymatic digestion.[20] Giannopoulou and Cimasoni reported the simple establishment of primary gingival fibroblast culture as they adhere to the culture plates and spread well to allow good proliferation without requiring any specific culture conditions.[21] Moreover, gingival fibroblasts are the major constituents of gingival tissues and function in maintenance as they are involved in the production

of extracellular matrix of tissue, both in disease and health.[22-23] Egusa et al. reported that through the process of efficient reprogramming of gingival fibroblasts, it is possible to make the gingiva a good source for induced pluripotent stem (iPS) cells in drug screening applications and for autologous cell therapy in the dentistry field.[24] Therefore, this led to the choice of HGF-1 in the current study.

Some studies have been carried out to demonstrate the beneficial effects of stingless bee honey on fibroblast cell previously. A study carried out by Nordin and colleagues reported that at the low dose of 0.024µg/ml, stingless bee honey extract possessed a beneficial effect on the dermal fibroblast viability and proliferation.[25] Another study by Malik et. al. demonstrated that stingless bee honey beneficially increased collagen type I expression and decreased MMP-1 expression during cellular aging of human dermal fibroblast cells.[26] Malaysian propolis collected by the stingless honey bee Trigona spp showed an overall positive effect on both fibroblast migration and proliferation assays compared to the control, and it followed a concentration-dependent curve with 250µg/ml being the most optimum concentration for cell migration and 500µg/ml for cell proliferation.[27]

In this study, the assessment of cell viability depended on the cytotoxic effect of Kelulut honey on HGF-1 cell line. A study on Sprague Dawley rats showed that Kelulut honey had chemopreventive properties against azoxymethane-induced colon cancer based on the reduction of total number of aberrant crypt foci and crypt multiplicity.[28] Similar studies were also performed by Pashinskiĭ and Gribel et al., who reported that honey possesses moderate anticolon cancer activity.[29] An in vitro study was also done to screen for the cytotoxic activity of different stingless bee products against five human cancer cell lines, namely, BT474 (ductal carcinoma and lung undifferentiated cancer), HepG29 (liver hepatoblastoma), KatoIII (gastric carcinoma), and SW620 (adenocarcinoma), where the crude extracts of stingless bee honey showed great cytotoxicity effect towards HepG2 cell line, while propolis crude extracts exhibited high cytotoxic effect towards all the human cancer cell lines.[30] A study was carried out by Nafi et al. on four stingless bee propolis ethanol extracts namely, Heterotrigona itama (H. itama), Geniotrigona thoracica (*G. thoracica*), *Lepidotrigona terminata* (*L. terminata*) and *Tetrigona apicalis* (*T. apicalis*) against triple negative breast cancer cells (MDA-MB-231), uterine leiomyosarcoma cells (SKUT-1) and cervical cancer cells (HeLa).[31] They concluded that *H. itama* produced the most active extract in terms of cytotoxicity and had the potential to be an antioxidant agent compared to propolis produced by other Malaysian stingless bee species. According to Borrelli et al.[32] caffeic acid phenethyl ester (CAPE) which is an important active component of honeybee propolis that possesses a plethora of biological activities, inhibited the development of azoxymethane-induced colonic aberrant crypt foci in the colon of rats. Besides, another study based on human sporadic colon cancer cell line, HCT116 treated with CAPE at serial concentrations reported that CAPE had antioxidant effect, biological and pharmacological functions including immunoregulation, anti-inflammatory, antiviral, antibacterial, and antitumor activities.[33] It was shown that the local presence of CAPE resulted in a significant delay in tumour formation in a mice model as well as significantly suppressed the proliferation of human HeLa cervical carcinoma cells in vitro which they attributed to the cytotoxic nature of the polyphenolic compounds.[34]

Compared to human cancer cell lines, there are also limited studies on the cytotoxicity of Kelulut honey on normal human cell lines. In the current study, the HGF-1 treated with Malaysian Kelulut honey showed high cell viability of more than 70% at all concentrations ranging from 3.125mg/ml to 200mg/ml and based on ISO 10993-5,[35] it can be inferred that Kelulut honey can was not cytotoxic at these concentrations. The statistical analyses found significant differences in the cell viability between concentrations of 200mg/ml - 3.125mg/ml at 24, 48 and 72 hours. The cell viability of the HGF-1 treated was generally higher at 48 hours than 72 hours. This could be due to the accumulation of waste products from the cells in the culture media over time. Moreover, a dose-dependent decrease in the cell viability was noticed; the higher the concentration of Kelulut honey, the lower the cell viability. The cell viability of HGF-1 was lowest at 200 mg/ml which were 75.87%, 81.17% and 77.47% at 24, 48 and 72 hours respectively, whereas the cell viability was highest at 3.125mg/ml which were 113.30%, 112.87% and 117.13% at 24, 48 and 72 hours respectively. The reduced cell viability at higher concentrations could be due to the increase in viscosity which could affect the medium and hinder the proliferation of cells. Moreover, it has been reported previously that different concentrations of honey could result in the change of osmolality of the media which can then negatively affect the growth of cells.[36] However, based on these fundamental results, further research needs to be carried out to exploit the potential of Malaysian Kelulut honey in clinical applications such as tissue engineering and regenerative medicine in the future.

CONCLUSION

This study showed that the cell viability of HGF-1 treated with Malaysian Kelulut honey was more than 70% at all concentrations ranging from 3.125mg/ml to 200mg/ml at three different time points, namely 24, 48, and 72 hours indicating that Malaysian Kelulut honey was not cytotoxic to HGF-1 cell line based on MTT assay under the test conditions.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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