The effect of Malaysian stingless bee, *Trigona* spp. honey in promoting proliferation of the undifferentiated stem cell

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Abstract. Stem cells provide various potential applications in regenerative medicine through its ability of self-renewal and differentiation. Among the various stem cells, dental pulp stem cells (DPSCs) have shown encouraging results in their ability to regenerate. Honey has been used in traditional culture as a natural medicine in supporting wound healing. Yet, very few studies on honey were conducted for its potential as a proliferative agent for stem cells. The aim of this study is to evaluate the stability of two *Trigona* spp. honeys (1 and 2) added in culture media and its proliferative effect on DPSCs. Both honeys were diluted with standard culture medium through dilution process to prepare the concentrations of 0.01%, 0.04%, 0.10% and 0.25%. DPSCs were treated with the diluted honeys for 24 hours. The proliferative activity was determined through the images taken using an inverted microscope for every six hours. In addition, the MTT assay was conducted to determine the cell viability of DPSCs when treated with both honey 1 and 2 at various concentrations. The results showed a stable culture media added with honey for three days and a dose-dependent proliferative effect of both *Trigona* spp. honey samples on DPSCs. Optimum proliferative effects were observed at 24 hours for both *Trigona* spp. honey 1 and 2 on DPSCs. The optimum concentration of *Trigona* spp. honey 1 was from 0.04% to 0.10% and *Trigona* spp. honey 2 was below 0.01%. It is concluded that *Trigona* spp. honey has a promising proliferative effect on DPSCs.

Keywords: *Trigona* spp. honey, mesenchymal stem cells, dental pulp, DPSCs, kelulut

INTRODUCTION

In recent years, many studies have focused on the possible application of stem cells to mend and regenerate body structures. By definition, stem cell is a cell that has the ability to divide (self-replicate) for indefinite periods throughout the

life of the organism (Chandross & Mezey, 2001). Stem cells are immature, unspecialised cells that have the potential to develop into many different cell lineages. For example, they can develop into mature cells that have characteristic shapes and

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specialised function, such as heart cells, skin cells, or nerve cells. Generally, stem cell research in regenerative medicine focuses on restoring the vitality and function of diseased and traumatised tissue (Noce *et al.*, 2014).

Mesenchymal stems cell (MSC) was obtained from the dental pulp of teeth. Dental pulp is a connective tissue that contains MSCs known as dental pulp stem cells (DPSCs) that is relatively easy to be obtained from the exfoliated primary teeth or extracted permanent teeth (Piva, Silva & Jacques, 2014) and is able to differentiate into different kinds of cells and tissues. As compared to bone marrow MSCs, DPSCs has higher proliferation rate, availability, and greater cell number (Noce *et al.*, 2014).

In the context of regenerative therapies, DPSCs hold great promise for cell therapy approaches in dentistry with studies already showing encouraging results in their ability to regenerate bone and periodontal tissues. Specifically, the identification of DPSCs promises a great potential in the regeneration of lost dental pulp and tooth tissues because of their ability to differentiate into dentin and pulp-like tissue (Eubanks *et al.*, 2014). Previous studies from Obeid *et al.* (2013) have shown that DPSCs are putative candidate cells for tooth and bone-tissue engineering. The identification of the MSCs capability in regenerating organised tooth-like structures has increased the interest in the potential use of postnatal stem cell-based therapies for dental tissue regeneration after trauma or disease.

Honey is a naturally available food with a long history of traditional use as an active medicinal compound in a large number of cultures. It contains a variety of phytochemicals with high flavonoid and phenolic content that become the main sources of high antioxidant activity of honey (Yao *et al.*, 2003). Various findings have proven that this natural product does not only exhibit antioxidant properties, but also immunoregulatory, chemopreventive, antiatherogenic, wound healing, and antimicrobial properties (Molan, 2006; Tsiapara *et al.*, 2009; Khalil *et al.*, 2010; Sherlock *et al.*, 2010).

In this respect, honey is well known for its healing potential. Understanding the healing potential in honey requires an understanding of the detail of its physico-chemical characterisation

and also an analysis of biological impacts at different concentrations/dilutions at different levels (e.g. cellular and molecular) (Chaudhary *et al.*, 2015). Research on the benefits of honey for health industry has been extensively conducted in many countries all over the world. However, there are few findings on the benefits of various honeys that exist in Malaysia, particularly *Trigona* spp. honey. In a comparison between the Tualang and the *Trigona* spp. honey, the latter has not been widely studied with only several findings on the effect of *Trigona* spp. propolis are reported. Thus, it is important to thoroughly study the benefits and potential contributions of the Malaysian stingless bee honey to the human health, especially as a proliferative agent.

Honey produced by stingless bee (*Trigona* spp.), which is also known as 'kelulut' in Malaysia, has been identified to have medicinal values. *Trigona* spp. honey is widely used by the aborigines and is considered to induce better performance than honeybee honey (Shahjahan *et al.*, 2007). The Malaysian Agricultural Research and Development Institute (MARDI) in Malaysia conducted research on this species and found that *Trigona* spp. consists of over 30 species in Malaysia. Among the species of *Trigona* spp. are *Trigona itama*, *Trigona apicalis*, *Trigona laeviceps*, and *Trigona thorasica* (Aquaponic Cultures Malaysia [ACM], 2012).

Trigona species are distributed throughout the tropical and subtropical parts of the Afrotropical, Australasian, Indo-Malayan, and Neotropical regions (Klasikorn *et al.*, 2005). Among the countries that have been identified to own *Trigona* spp. are Indonesia (37 species), Thailand (20 species), Vietnam (9 species), India (3 species), Taiwan (1 species) and Malaysia (more than 30 species) (ACM, 2012).

Healing with diluted honey rather than its raw form is also proposed considering the fact that a low concentration of hydrogen peroxide in a healing agent has bactericidal effect but is not toxic on mammalian cells (Molan, 2006). Therefore, knowledge on the optimum dilution of honey is imperative in order to determine its maximum healing efficiency. Impacts of specific honey dilutions on epithelial cell population *in vitro* also need to be investigated.

Herein, this study aimed to investigate the potential role of *Trigona* spp. honey in proliferative

effect on dental pulp stem cells (DPSCs). Hence, this study is designed to evaluate the differential dosage effect of *Trigona* spp. honey. It is hypothesised that honey from this species is able to increase the proliferative activity of DPSCs in a dose- and time-dependent manners.

MATERIALS AND METHODS

Honey procurement. *Trigona itama* species honey from two different places were procured from Assoc. Prof. Dr. Muhammad bin Ibrahim, from Department of Nutrition Science, Kulliyyah of Allied Health Science, International Islamic University Malaysia (IIUM). It was available in the market and the places were not to be revealed in this study in order to avoid any conflict of interest.

Dental pulp stem cells (DPSCs) procurement. Human DPSCs (Passage 10, P10) were obtained from Dr. Shamsul Azlin (which was derived from his lab), The Department of Biology, University Malaya. The validations of cells were done by identifying the protein specific markers of CD105 and CD90 with fibroblast-like shape of the cells (Helena, Alencar, & Kitten, 2011; Nikolić *et al.*, 2011). The cells were cultured and maintained in Molecular and Cellular Biology Lab, Integrated Centre for Research Animal Care and Use (ICRACU), IIUM.

Serial dilution of honey. The sample was kept in a tightly packed and sterilised container to avoid contamination until use and was certified by the Ministry of Health Malaysia (MOH) for marketing purposes. The absolute honey (100%) with the initial pH 3.3 and moisture content of 12% was then diluted at the concentrations of 0.01% (v/v), 0.04% (v/v), 0.10% (v/v), and 0.25% (v/v) with culture medium. The honey mixtures were prepared 24 hours before being tested to cell lines and stored at 4°C until use.

Media stability test. Each media was prepared with different concentrations of *Trigona* spp. honey 1 and 2. About 5 ml of each concentration were delivered into T25/25 cm² cell culture flasks. Then, the media added with honey was kept in 5% CO₂ incubator at 37°C for three days. Images

were taken before and after three days in the incubator using EVOS XL CORE (Thermo Fisher Scientific, USA).

Cell culture. DPSC lines were propagated in Dulbecco's modified Eagle's medium (DMEM) in three different cell culture flasks, all supplemented with 10% fetal bovine serum (FBS) and 5% penicillin/ streptomycin, and maintained at 37°C in 5% CO₂ incubator.

Proliferation test. The proliferation test was performed based on the work of Fauzi *et al.* (2011) using haemocytometer with modification. In order to perform the proliferation assay for DPSC lines, at least 5,000 or 5×10^3 cells per well are recommended. The cells were seeded in 12-well plate at three different cells density; 5×10^3 cells, 10×10^3 cells, and 15×10^3 cells well. The cells were incubated overnight at 37°C with 5% CO₂ and treated for 24 hours at four different concentrations of both *Trigona* spp. honey samples 1 and 2 (0.01%, 0.04%, 0.10% and 0.25% respectively). Images were taken using an inverted microscope for every 6 hours interval until 24 hours of treatment at 10X magnification.

Evaluation of cell viability in media added with honey and control. In this study, we conducted the cell viability by using (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide [MTT]) colorimetric assay. DPSC cell lines were seeded at a density of 5000, 10000, and 15000 cells/well in a 96-well plate. The best-seeded number of cells was analysed in this paper. The three densities were chosen based on the optimum densities obtained from the proliferation test. After an incubation period of 24 hours, the cells were washed with phosphate buffered saline (PBS). The different concentrations (0.01% (v/v), 0.04% (v/v), 0.1% (v/v), and 0.25% (v/v)) of honey were added into the cells and incubated at 37 °C and 5% CO₂ for 24 hours. A 20 µl of MTT reagent (0.5 mg/ml) was dispensed into each well and incubated for 2 hours. Subsequently, MTT reagent was aspirated and 200 µl of dimethyl sulfoxide (DMSO) was added to solubilise the formazan. This technique was performed in a dark room. The absorbance was recorded using spectrophotometer under a wavelength of 450 nm. Each experiment was

replicated three times for each concentration and result obtained was compared with the control.

Data analysis. All proliferative activities of DPSCs were observed through the images taken by an inverted microscope (EVOS XL CORE, Thermo Fisher Scientific, USA) for 24 hours with the intervals at 0, 6th, 12th, and 24th hour. Images obtained were observed and compared with the control. Comparisons within the groups of data were analysed using ANOVA and the probability of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of *Trigona* spp. honey on culture media. Both *Trigona* spp. honey samples of 1 and 2 were tested for their stability with media containing DPSCs at the concentration of 0.01%, 0.04%, 0.1% and 0.25% v/v. Results showed that after 72 hours, all media did not change its appearance or colour as compared to the control medium (Figure 1).

Figure 1. Comparison of media stability based on different concentrations of *Trigona* spp. honey 1 and 2. **Effect of *Trigona* spp. honey on DPSC proliferation.** All the four concentrations showed relative proliferation activities when compared to the control.

The obtained proliferative activities varied not only in concentrations and time, but also between the two different samples (*Trigona* spp. honey 1 and 2). The observations were made based on the result of the images captured which are more on the qualitative result and yet to be confirmed by MTT assay for quantitative result.

Figure 2 shows the overall images of 15×10^3 DPSCs in 24 hours based on the different concentrations of *Trigona* spp. honey 1 (result for 5×10^3 and 10×10^3 are not shown). The best-seeded number of DPSCs cultured with *Trigona* spp. honey 1 was 15×10^3 and showed an increase in proliferation rate from 0.04% (v/v) to 0.10%

(v/v) concentration. However, the proliferation rate was slower at a higher concentration of 0.25% (v/v) (Figure 2). Meanwhile, in DPSCs treated with *Trigona* spp. honey 2, higher proliferation rate was only observed at the concentration of 0.01% (v/v) (Figure 3). Therefore, higher proliferation rate was achieved 24 hours after being treated with both *Trigona* spp. 1 and 2. Thus, it can be suggested that both *Trigona* spp. honey 1 and 2 are able to induce a significant proliferation activity of DPSCs in their respective range of concentrations.

Figure 2. Overall images 15×10^3 DPSC in 24 hours based on different concentrations of *Trigona* spp. honey 1. Magnification X20.

Figure 3. Overall images 15×10^3 DPSC in 24 hours based on different concentrations of *Trigona* spp. honey 2. Magnification X20.

Evaluation of honey on cell viability. To confirm the result obtained from the proliferation test, MTT assay was carried out to look at the optimum proliferation effects of both honeys. Only the concentrations that gave higher proliferative activity as compared to the control were chosen for cell viability test, which are Type 1 Honey 0.04% and 0.1%, and Type 2 Honey 0.01% at 15×10^3 seeded cell number. Figure 4 demonstrated that the optimum proliferation effects of *Trigona* spp. honey 1 are within the concentration 0.04% (v/v) and 0.10% (v/v). Meanwhile, for *Trigona* spp. honey 2, the optimum value is at concentration 0.01% (v/v) (Figure 5). In addition, both *Trigona* spp. honey 1 and 2 showed that the best-seeded number of DPSCs cultured was 15×10^3 cells/well.

Figure 4. Effects of media added with *Trigona* spp. honey 1 on DPSCs at seeding density of 15,000 and treated at the concentrations of 0.04% and 0.10%. Data are presented as $\pm$ s.d. and represent of three experiments. Significance at p-value is less than 0.05.

Figure 5. Effects of media added with *Trigona* spp. honey 2 on DPSCs at seeding density of 15,000 and treated at the concentration of 0.01%. Data are presented as $\pm$ s.d. and represent of three experiments. Significance at p-value is less than 0.05.

DISCUSSION

Honey is a very complex product because its chemical properties and composition depend not only on the nectar-providing plant species, but also on other factors such as the bee species, geographic area, season, mode of storage, harvest conditions, and even the interactions between the chemical compounds and enzymes in the honey. It is known that the accumulation of phytochemicals in honey depends on climatic conditions (sunlight, moisture), soil characteristics, and other factors. Therefore, it is reasonable to believe that the differences between honey from country to country appear due to the different compositions of pollen or nectar, which have the greatest influence on the chemical composition of honey. For instance, essential oil composition is very dependent on the geographical location even for the same plant species thus suggesting that even the same floral origin honey composition may be quite different (Kaškonienė & Venskutonis, 2010).

Most of the available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes colour as pH is changed due to the metabolites released by the cells. At low pH levels (increased acidity), phenol red turns the medium to yellow, while at higher pH levels it turns the medium to purple. The medium is bright red for pH 7.4, the optimum pH

value for cell culture (Arora, 2013). From Figure 1, images showed that there were no changes in the colour of DMEM media without DPSCs after being incubated for three days. This showed that the substances from both *Trigona* spp. honey 1 and 2 did not alter the stability of the media.

Media supplements are the growth media recommended for certain cell lines that require additional components which are not present in the basal media and serum. These components and supplements help to sustain cell proliferation and maintain normal cell metabolism (Arora, 2013). The additional substances from both *Trigona* spp. honey samples did not alter or change the standard culture media of DPSCs by providing a stable colour of pH 7.4.

The addition of *Trigona* spp. honey might affect the osmolality of the culture media as well as its stability. For most of the cell lines, optimal osmolality for culture media should be between 260 mOSM/kg and 320 mOSM/kg (Arora, 2013). Thus, the change of the culture media osmolality will affect the DPSCs proliferative activities. This factor may be the reason why *Trigona* spp. honey 1 showed proliferative activity at 0.04% and 0.10% concentration, while *Trigona* spp. honey 2 showed only active proliferative activity at 0.01% concentration. Both of the *Trigona* spp. honey samples may have different osmolality due to the different chemical compositions and concentrations since the addition of supplements from honey can change the osmolality of the complete growth media that can negatively affect the growth of cells. It is always best to recheck the osmolality after supplements are added because it is essential in ensuring that the cells can keep surviving and proliferating. High osmolality could affect the water from the cells to rush out, resulting in shrinkage. Therefore, the addition of the supplements in the honey might alter the osmolality that can affect the proliferation of the cells.

MTT assay was done to determine the best seeding number for DPSCs to proliferate at optimum range. Three different ranges of cell seeding number were tested in this study; namely 5×10^3 , 10×10^3 , and 15×10^3 cells/well. The best seeding number of DPSCs was chosen by comparing the overall triplicate of the DPSCs through proliferation test and viability test using the MTT assay. Cell density or cell seeding

number is known to control the rate of proliferation of cells in culture. The transport of small nutrients also depends on the cell density. It decreases when cell density increases and vice versa. Increases in the uptake of low molecular-weight nutrients are among the early events associated with the initiation of cell growth (Piedimonte, Borghetti & Guidotti, 1982).

From previous studies, Xia *et al.* (2011) stated that a wide range of endothelial cell seeding densities from 4×10^3 to 2×10^5 cells/cm² have been used for *in vitro* studies on cell-biomaterial interaction. Stimuli from the neighbouring cells via the interaction of cell surface receptors and secreted growth factors or cytokines are strongly dependent on the cell density. When the cell density is low, direct cell-cell contacts are limited and cell-biomaterial interaction is expected to be pre-dominantly influenced by cell-substrate contact. Compared to the low seeding density, cell-cell interaction at high seeding density is more extensive, which could contribute to cell attachment signalling activation through crosstalk between cell-substrate and cell-cell adhesion (Xia *et al.*, 2011). Thus, this previous finding is similar to the present study in which the number of seeding cells was found to influence the cells' proliferation activity.

Stem cells exhibit variable differentiation and proliferation characteristics of the same cell line if it is grown in different types of serum and can also present different phenotypes if cultivated in different conditions. Nevertheless, the cellular requirement for a specific micronutrient is directly correlated with the cell type, the rate of cell growth, and the stage of cell differentiation (Arigony *et al.*, 2013).

Based on Figures 2 and 3, at concentrations between 0.04% (v/v) and 0.10% (v/v) for honey 1, the best proliferation time for DPSCs was 24 hours. Similarly, the best time for proliferation is 24 hours for *Trigona* spp. honey 2 at the concentration of 0.01% (v/v). Xia *et al.* (2011) stated that as cell density increases, cell-cell interaction also increases and it is expected to profoundly influence cellular responses to biomaterials.

The composition of honey is rather varied and depends primarily on its floral source, however, certain external factors, such as seasonal environmental factors and processing methods

also play a role. The varying proliferative effect of honey may be due to the different substances and depends on the botanical origin of honey. For example, different antimicrobial chemicals were found on different bee honey; methylglyoxal was discovered to contribute to the activity of New Zealand's Manuka honey, bee defensin-1 was detected in a Dutch honey, and melanoidins were identified in Canadian honeys (Moussa *et al.*, 2012).

Phenolic compounds may be one of the factors that increase the proliferative activity of DPSCs as compared to the standard culture media. The compounds, such as flavonoids, caffeic acid, di-hydroxy benzoic acid, cinnamic acid, and ferulic acid are found in different types of honey at various concentrations depending on the location and botanical sources (Yao *et al.*, 2003; Jaganathan & Mandal, 2009; Tsiapara *et al.*, 2009). As for the stingless bee honey, specifically *Trigona* spp. honey, it is found that the honey contain free phenolic acids, including phenylpropanoic acid, benzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, vanillic acid, protocatechuic acid, and p-coumaric acid (Roowi *et al.*, 2012). In the present study, there are possibilities that one or more of the phenolic acids or compounds mentioned above might be the component that contributed to the proliferative effect on DPSCs. Thus, further study to identify the compounds and their effects is needed.

Other factors that influence the proliferation rate of DPSCs also need to be taken into consideration. An extensive review of the antimicrobial activity of honey showed it to be derived from high sugar content, low water content, acidity, the generation of hydrogen peroxide on dilution, and phytochemical components (Moussa *et al.*, 2012).

In this study, the MTT assay was used to observe the optimum concentration of both honey toward the cell proliferation effect of DPSCs. Based on the data that we had, at 15×10^3 seeded cell number, the optimum concentration of *Trigona* spp. honey 1 is between 0.04% (v/v) and 0.10% (v/v), as compared to the control. In a comparison with the control, the optimum concentration of honey 2 is 0.01% (v/v). Therefore, when the concentration is less than the optimum value, the honey might show slow

proliferation effects. However, if the concentration of honey is greater than the optimum value, it might cause a reduction in the cell proliferation activity.

CONCLUSION

This study demonstrated that *Trigona* spp. honey 1 and 2 have a promising proliferative effect on DPSCs. This honey was found to not have any effect towards the media stability within three days of incubation. It also increased the proliferation rate of DPSCs as early as 24 hours with 0.04% and 0.10% concentrations of *Trigona* spp. honey 1 and only 0.01% concentration of *Trigona* spp. honey 2 as compared to the control.

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