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Modulation of Human Bone Marrow Mesenchymal Stem Cells (BMMSC) by *Nigella sativa* and *Trigona* Honey: An *in vitro* Study

(Modulasi Sel Stem Mesenkima Sumsum Tulang Manusia (BMMSC) oleh *Nigella sativa* dan Madu *Trigona*: Suatu Kajian *in vitro*)

Masniza Muhamad Lasim[1], Nurul Farihah Samsudin[1], Zetty Nadia Mohd Zain[2], Hayati Abdul Rahman[2], Nur Syahrina Rahim[2], Asral Wirda Ahmad Asnawi[2] & Nur Fariha Mohd Manzor[1,*]

[1]*Department of Medical Sciences I, Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, 71800 Nilai, Negeri Sembilan, Malaysia*
[2]*Department of Medical Sciences II, Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, 71800 Nilai, Negeri Sembilan, Malaysia*

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ABSTRACT

Maintenance of bone marrow mesenchymal stem cells (BMMSC) numbers is crucial for tissue repair and regeneration since adult stem cells are often limited in number, expansion capacity and lifespan. It is believed that certain types of foods are beneficial in the preservation and stimulation of stem cells throughout life. Black seeds and stingless bee honey are among the widely investigated functional food with general health promoting benefit. We aim to explore the proliferative, anti-apoptosis and anti-senescence effect of *Nigella sativa* and *Trigona* honey on BMMSC. Cell proliferation was evaluated using MTT assay. We performed flow cytometric analysis to verify stem cell surface markers while quantitative PCR was done to measure the relative expression of target genes. Results showed favourable concentration dependent enhancement of cell proliferation by 1 to 10 ug/mL *Nigella sativa* and 0.097% to 0.195% *Trigona* honey. Observation on BMMSC's morphology and surface markers expression revealed no alteration on BMMSC stemness properties. In addition, gene expression analysis supported that there was a significant ($P<0.05$) increase in proliferation related gene, β-Catenin, and lower expression of apoptotic- and senescence-related gene, BAX and p21, respectively. These preliminary findings suggest a promising effect of *Nigella sativa* and *Trigona* honey on stem cell proliferation.

Keywords: Apoptosis; black seed; bone-marrow mesenchymal stem cells; proliferation; senescence; stingless bee honey

ABSTRAK

Pengekalan bilangan sel stem mesenkima sumsum tulang (BMMSC) adalah penting untuk pembaikan dan penjanaan semula tisu kerana sel stem dewasa selalunya terhad dalam bilangan, kapasiti pengembangan dan jangka hayat. Jenis makanan tertentu adalah dipercayai bermanfaat dalam pemeliharaan dan ransangan sel stem sepanjang hayat. Habbatus sauda dan madu lebah kelulut adalah antara makanan bermanfaat terhadap kesihatan yang dikaji secara meluas. Penyelidikan ini bertujuan untuk meneroka kesan proliferatif, anti-apoptosis dan anti-penuaan *Nigella sativa* dan madu *Trigona* terhadap BMMSC. Proliferasi sel telah dinilai menggunakan ujian MTT. Kami menggunakan analisis aliran sitometriks untuk mengesahkan penanda permukaan sel stem manakala PCR kuantitif dilakukan untuk mengukur pengekspresan relatif gen sasaran. Keputusan menunjukkan proliferasi sel yang menggalakkan dalam kepekatan sebanyak 1 hingga 10 ug/mL *Nigella sativa* dan 0.096 hingga 0.195% madu *Trigona*. Pemerhatian pada morfologi dan ekspresi penanda permukaan BMMSC menunjukkan tiada perubahan pada sifat BMMSC. Tambahan pula, analisis pengekspresan gen menyokong bahawa terdapat peningkatan yang ketara ($P<0.05$) dalam gen proliferatif, β-Catenin manakala gen apoptosis dan gen penuaan, BAX dan p21, masing-masing menunjukkan ekspresi yang lebih rendah. Penemuan awal ini mencadangkan kesan memberangsangkan *Nigella sativa* dan madu *Trigona* terhadap proliferasi sel stem.

Kata kunci: Apoptosis; Habbatus sauda; madu lebah kelulut; penuaan; proliferatif; sel stem mesenkima sumsum tulang

INTRODUCTION

Chronic degenerative diseases for instance diabetes, heart disease, obesity, chronic respiratory diseases, neurodegenerative diseases, and cancer are relentless non-infectious conditions characterized by the gradual deterioration of tissue or organ function over time, often resulting in impaired mobility, cognitive decline, or organ failure (Di Renzo et al. 2023). The progress of degenerative diseases was strongly correlated to age factor (Franceschi et al. 2018; Hou et al. 2019). On the other angle, it was well-documented that ageing could influence the number of stem cells available for self-renewal hence compromising the reparative capacity of tissues in the human body and could contribute to the progress of disease (Ahmed et al. 2017). Hence, the maintenance and modulation of stem cells capacity have emerged as important aspects in regenerative medicine. The unique metabolic and nutritional needs of stem cells relative to differentiated cells and the impact of nutritional types on quantity and quality of stem cells available for tissue renewal have become the topic of interest for many researchers. Previous studies have suggested the stem cell modulation effects of certain type of diet including the practice of intermittent fasting (Puca et al. 2022) and Mediterranean diet, less intake of glucose (Junaid et al. 2021) and lipid as well as high intake of polyphenols-rich foods such as grape and olive oils (Puca et al. 2022). A number of studies have shown promising modulation effect of functional food on stem cells. For instance, green tea extracts were reported to significantly increased the number of circulating endothelial progenitor cells (EPCs) in healthy human subjects (Kim et al. 2017). Another study demonstrated that blueberry and strawberry extracts promoted the proliferation and differentiation of neural stem cells (NSCs), suggesting a potential role in brain health and neurogenesis (Rutledge et al. 2019). Our previous research also demonstrated a positive stimulation of bone marrow mesenchymal stem cells proliferation by date palm fruit extracts (Masniza et al. 2020). In this present study we are attracted to investigate on two widely proposed functional foods, the black seeds and honey from the stingless bee of *Trigona* sp.

Nigella sativa (NS) or commonly known as black seed is a species of medicinal plant under botanical family of *Ranunculaceae*. Extensive studies have shown the therapeutic potential of *Nigella sativa* as anticancer (Randhawa & Alghamdi 2011), anti-inflammatory (Gholamnezhad, Keyhanmanesh & Boskabady 2015), hypoglycemic agent (Kaatabi et al. 2015) and anti-hypertensive (Dehkordi & Kamkhah 2008). Previously, some studies which were done on a few types of cells including splenocytes (Majdalawieh, Hmaidan & Carr 2010) and bone cells (Prasetyaningtyas et al. 2016) have demonstrated the proliferative effect of *Nigella sativa*.

On the other hand, honey is a golden sweet liquid derived from the nectar of flowers collected by bees. *Trigona* species, also known as '*Kelulut*' locally is one of the stingless bee genera found in Malaysia. *Trigona* honey (TH) has received much attention for its pharmacological properties such as antibacterial and chemo-preventive properties (Zainol, Mohd Yusoff & Mohd Yusof 2013). Recent discoveries on animal model for menopause have demonstrated the proliferative effect of Tualang honey (*Apis* genera) in uterus, vaginal epithelium, tibia bones and hippocampus (Al-Rahbi et al. 2014; Zaid et al. 2010). However, there are still limited literatures and research done on *Trigona* honey on mesenchymal stem cells.

Given the influence of diet and nutrition on stem cells and accumulated evidence of health benefits of *Nigella sativa* (black seed) and honey, it is worth to explore the mechanism of stem cell alteration by these selected foods. Thus, we focused our research on the effects of these foods on human bone marrow mesenchymal stem cell (BMMSC), an adult stem cells which play important roles in regeneration process of injured tissues. MSC have the ability to self-renew (Al-Rahbi et al. 2014), differentiate into different cell types (Sasaki et al. 2008), express paracrine effect (Shen et al. 2015), modulate immune response (Yi & Song 2012) and believed to migrate to injury site to repair some pathological conditions such as bone fracture (Alm et al. 2010), liver injury (Chen et al. 2010) and brain injury (Zhang et al. 2013). With regards to all these crucial functions of BMMSC, it is important to maintain BMMSC numbers for normal tissue regeneration. It was highlighted that the understanding of molecular relationship between diet and stem cell properties would drive the efforts towards identifying potential diet-based strategies to improve overall health and prevent degenerative diseases (Puca et al. 2022). Hence, investigation on BMMSC changes upon treatment with potential foods will further improve our understanding on their benefit in the preservation and stimulation of stem cells. In this study, we investigated the effect of *Nigella sativa* and *Trigona* honey on BMMSC proliferation-, apoptosis-, senescence- and stemness-associated markers.

MATERIALS AND METHODS

Trigona HONEY ANALYSIS

A sample of *Trigona* honey was send to a food quality and safety laboratory service, UNIPEQ, Universiti Kebangsaan Malaysia (UKM) for analysis of sugar profile, total ash, moisture, and free acidity.

PREPARATION OF *Nigella sativa* AND *Trigona* HONEY

The protocol used for aqueous extraction of *Nigella sativa* was according to methods by Ab Rahman, Abdul Razak and Mohd Bakri (2014) with slight modification. 100 g of *Nigella sativa* seeds was weighed, washed, and blended in 1,000 mL of distilled water. Debris was removed by filtering the extract with gauze followed by a filter paper (Whatman #1). The concentrate was then dispensed into several 50 mL falcon tubes and stored in -80 °C overnight. The O-ring of the tubes were covered with parafilm and pricked few times. The extract was then sent to Institute of Bioscience, Universiti Putra Malaysia (UPM) for freeze-drying process. Meanwhile, *Trigona* honey was ready to use. Trigona honey was obtained from local supplier in Selangor. Dry extract of *Nigella sativa* and liquid form *Trigona* honey were diluted in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA), 1% antibiotic antimycotic (AA), 2% FBS (Gibco, USA) or without FBS and filtered using 0.45 uM filter for cell treatment.

CELL PROLIFERATION ASSAY

BMMSC were seeded in a 96-well flat-bottom plate at 2,500 cells/well in DMEM medium supplemented with 10% FBS and 1% AA. Once ready, the plates were placed in a humidified incubator at 37 °C and 5% CO_2. After 24 h, the media were removed and replaced with new media supplemented with 2% FBS or without FBS and enriched with different concentration (ug/mL) of *Nigella sativa*; 1, 10, 25, 50, 75, and 100 and different percentage (v/v) of *Trigona* honey; 0.097, 0.195, 0.39, 0.78, 1.56, 3.125, and 6.25. The plates were incubated for 24 h, 48 h, and 72 h and the cell viability were determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Following incubation, the media were replaced with 100 µL of DMEM, added with 10 µL MTT reagent and then incubated for 3-4 h. Lastly, 100 µL of dimethyl sulphoxide (DMSO) were added and the optical density (O.D) were measured at a wavelength of 570 nm using the microplate reader (Tecan Infinite M200 Pro). The test was carried out in triplicate and repeated three times (n=3) for standardization and reproducibility. The percentage of BMMSC viability was calculated based on the formula (Equation 1) and the result was presented as mean ± standard error of mean (SEM).

$$\text{Cell viability (\%)} = \left[\frac{(Absorbance_{(sample)} - Absorbance_{(blank)})}{(Absorbance_{(control)} - Absorbance_{(blank)})}\right] \times 100\% \quad (1)$$

BMMSC SURFACE MARKER VERIFICATION

After treated with selected concentration of *Nigella sativa* and *Trigona* honey for 48 h, the cells were washed and added with cell detachment solution (Accutase®) for cell detachment. Once the cells had appeared rounded under the microscope, the flask were gently tapped to detach the remaining cells. Cell suspensions were then transferred into falcon tube, centrifuged, and resuspended in 200 µL of staining buffer. BMMSC surface markers were verified using Human Mesenchymal Stem Cell Verification kit (R&D System) following the manufacturer's instruction. 100 µL of cell suspension were transferred into 2 flow cytometry tubes; unstained and stained. For the stained tube, 10 µL of each positive antibody (CD90, CD73, and CD105) and 10 µL of the negative marker cocktail (CD45, CD34, CD11b, CD79A, and HLA-DR) were added into a tube of cells and incubated for 30 to 45 min at room temperature in the dark. Next, cells were washed with 2 mL of staining buffer to remove any excess antibody. The final cell pellet and the unstained tube were resuspended in 400 µL of Staining Buffer for flow cytometric analysis (BD Facs Canto II).

TOTAL RNA EXTRACTION, AND REVERSE TRANSCRIPTION

Total RNA from the treated cells was extracted using TRI reagent according to the manufacturer's instruction (Molecular Research Center). Briefly, the cells were lysed directly in the culture media with 1 mL of TRI reagent (per 10 cm^2 surface area) followed by 5 min incubation at RT for complete dissociation of nucleoprotein complexes. 0.2 mL chloroform was added and centrifuged at 12,000 g, 4 °C for 15 min. Then, 0.5 mL of isopropanol was added to the separated colorless upper phase and centrifuged at 12,000 g for 8 min. A gel-like, white pellet at the bottom of the tube was washed with 1 mL of 75% ethanol and was dissolved in 20 µL of RNase-free water. The concentration and purity of the RNA was measured by Nanodrop.

The complementary DNA (cDNA) was then synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Thermo Fisher Scientific) according to the protocol recommended by manufacturer as follows: 1 µg of template RNA, 10 µL of 2X RT reaction mix, 2 µL of RT enzyme mix and DEPC-treated water was toped up in a 20 µL reaction. The samples was incubated in a thermocycler for 10 min at 25 °C, 5 min at 85 °C, terminated and chilled on ice before 1 µL (2 U) of *E. coli* RNase H was added into the tube. The samples were incubated again at 37 °C for 20 min.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qPCR) ANALYSIS

Quantitative PCR (qPCR) amplification was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on the AriaMx Real Time PCR machine (Agilent Technologies). Primer sequences were adapted from previous studies (Table 1) and made by 1st BASE Company. PCR program was set up as follow: 50 ℃ for 2 min, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s, 60 ℃ for 15 s and 72 ℃ for 1 min. TATA box binding protein (TBP) was used as reference gene. Melt curves were analysed and amplification efficiency (E) was calculated from an individual PCR reaction by Real-time PCR Miner (Zhao & Fernald 2005; http://www.miner.ewindup.info/). Efficiency corrected quantification was included in the calculation of mRNA relative expression using Pfaffl method (Pfaffl 2001).

STATISTICAL ANALYSIS

Data obtained from experiment were compared to control and the results were statistically analysed by SPSS version 20 using non-parametric test, Mann-Whitney test. The normality of the data obtained from the experiment was assessed using the Shapiro-Wilk test. The results indicated that the data did not follow a normal distribution ($p < 0.05$) thus differences between groups were statistically analysed by SPSS version 20 using non-parametric test, Mann-Whitney test. Results were expressed as mean ± standard error of mean (SEM).

RESULTS AND DISCUSSION

Trigona HONEY ANALYSIS

The quality of *Trigona* honey was determined by its physicochemical properties; ash, moisture, free acidity, reducing sugar and sucrose. All parameters showed high value except for ash and maltose which is not detected in the sample (Table 1). Recently, the Ministry of Science, Technology, and Innovation (MOSTI) via the Department of Standard Malaysia has developed Malaysian Standard (MS) 2683 *Kelulut* (Stingless bee) honey - Specification following the infusion of artificial honey in the market which affect consumers' confidence on Malaysian honey products. Based on the result of *Trigona* honey analysis, all parameters are in agreement with the standard (supplementary files) except for sucrose. However, a slightly higher content of sucrose in this study could be due to collection of honey before ideal maturation time (Batista de Sousa et al. 2016).

CELL PROLIFERATION ASSAY

In general, treatment by *Nigella sativa* extract for 24 h was not enough to induce the proliferative activity of BMMSC. After 48 h of treatment, the cell viability increased starting from 1 ug/mL to 50 ug/mL of *Nigella sativa* without FBS and small increase is observed at 10 ug/mL to 25 ug/mL of *Nigella sativa* supplemented with 2% FBS. Meanwhile, BMMSC showed decreasing proliferative activity after treated with *Trigona* honey as the concentration and time

TABLE 1. Physicochemical properties of *Trigona* honey

Parameter, Unit	Result
Ash, g/100 g	0.3
*Moisture, g/100 g	28.1
Free Acidity, meq/1000 g	158.7
*Reducing sugar, g/100 g	
- Fructose	31.0
- Glucose	27.4
- Maltose	N.D (<0.01)
*Sucrose, g/100 g	11.6

of treatment increased. Only 0.097% and 0.195% of honey enhanced the cell viability for both groups. At the concentration of 3.125% and 6.25%, honey supplementation significantly decreased the BMMSC viability (Figure 1).

From the results, it showed that the aqueous extract of *Nigella sativa* had moderately increased BMMSC proliferation. This was supported by previous findings which showed the proliferative effect of *Nigella sativa* on rat pancreatic β-cells (Albajali et al. 2011), balb/c mice bone marrow cells (Ghonime et al. 2011) and human gingival fibroblast (Ghonime et al. 2011). Thymoquinone and essential fatty acids in *Nigella sativa* was claimed by several studies to be responsible in stimulating the cells proliferation (Eid et al. 2017; Majdalawieh, Hmaidan & Carr 2010).

Meanwhile, the proliferative effect of *Trigona* honey showed high and significant BMMSC proliferation compared to *Nigella sativa*. A review paper done by Abd Jalil, Kasmuri, and Hadi (2017) showed that protocatechuic acid (PCA), 4-hydroxyphenylacetic acid and cerumen in stingless bee honey possess proliferative and antioxidant properties. To the best of our knowledge, this is the first study to describe the proliferative effect of *Trigona* honey on stem cells. Overall, this finding supports the proliferative effect of *Trigona* honey using minimal concentration but not seen in higher concentrations of honey. There was a similar study done using *Tualang* honey on human corneal epithelial progenitor cells treated for 48 h which showed reduced proliferation after the concentration of honey was increased to 3.33% (Tan et al. 2016). Both findings could be due to high glucose content in honey (Kek et al. 2017) which impaired the stem cell functions, induce apoptosis and cell senescence (Li et al. 2007; Stolzing, Coleman & Scutt 2006).

MORPHOLOGY

The morphological features of BMMSC were similar in treatment groups and control. The cells proliferated in a homogenous fibroblast-like cells morphology (elongated spindle) with enlarged cell bodies interconnected with various lengths and sizes of cytoplasmic projections (Figure 2). BMMSCs in the treatment group maintain their characteristic spindle-shaped morphology and typical size or aspect ratio with a well-defined cytoplasm and prominent nuclei. Senescent features of MSC such as enlarged, flattened, irregular nucleus and increased cytoplasmic granules and vacuoles were not observed in all groups. The cells did not exhibit degenerative features associated with increased cellular stress and apoptosis including cytoplasmic vacuolization, nuclear condensation and formation of apoptotic bodies.

BMMSC SURFACE MARKER

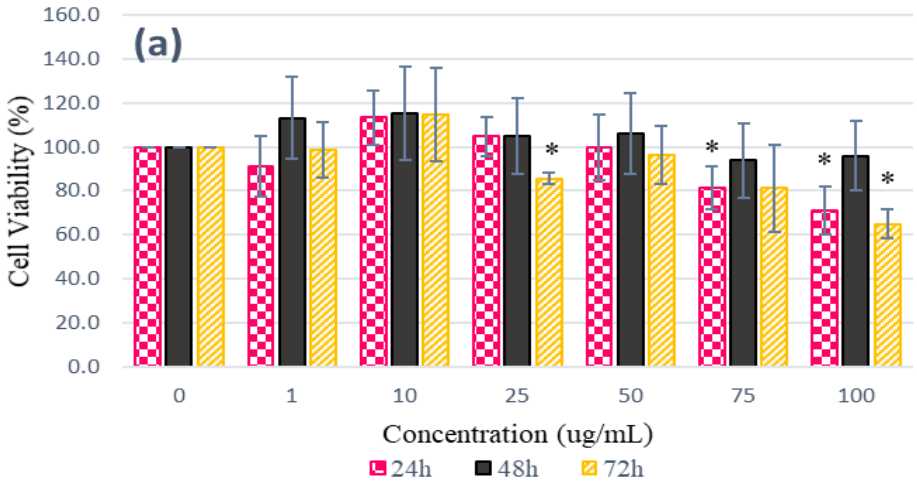
Table 2 depict positive expression of MSC-associated surface markers in range between 80%-92%. Although treatment by *Trigona* honey without FBS showed a slightly lower expression of CD73 and CD105, the statistical analysis showed no significant difference between treated groups and control group. Treatment of *Nigella sativa* and *Trigona* honey does not alter the BMMSC stemness in term of morphology and cell surface marker. The morphology of treated BMMSC illustrates similar features with control group and previous study (Li et al. 2015). High expression of CD73, CD105, and CD90 and lack in expression of other markers such as CD45, CD34, CD14, or CD11b proved that BMMSC retained its stemness properties after treated with the prophetic foods (Dominici et al. 2006).

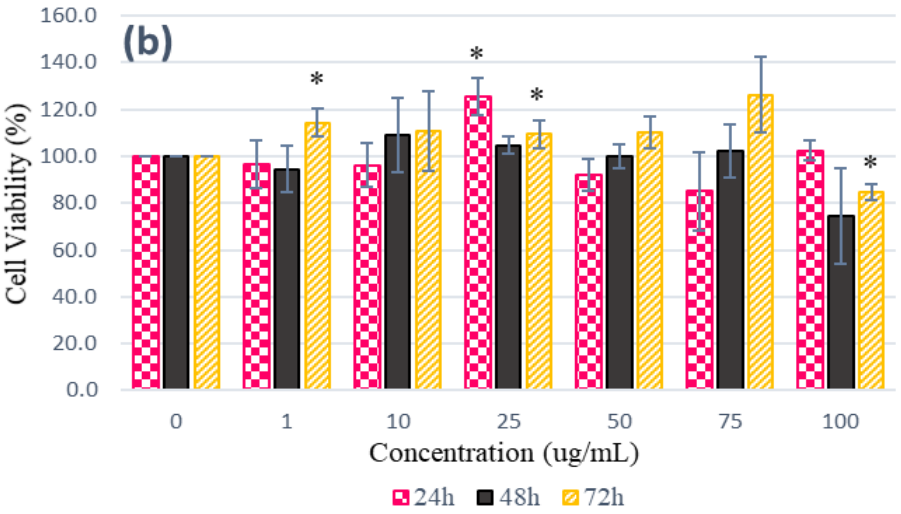
QUANTITATIVE REAL-TIME PCR

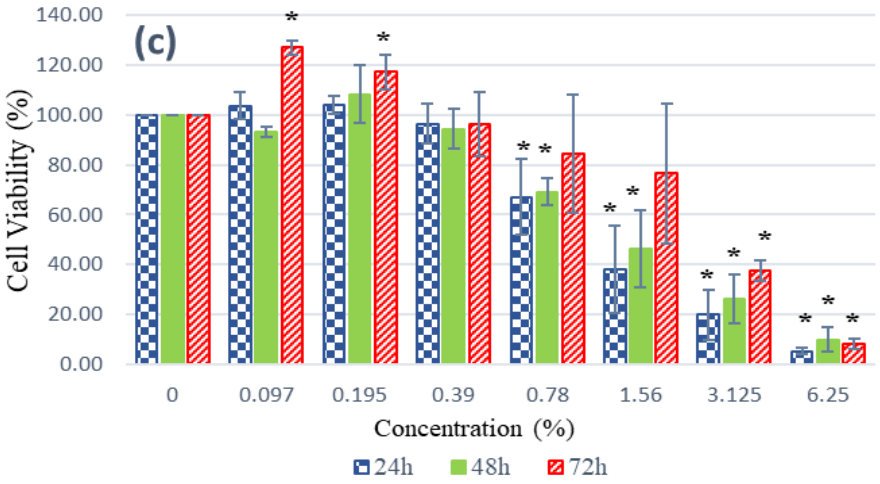
PROLIFERATION-ASSOCIATED GENES

Nigella sativa and *Trigona* honey supplemented with 2% FBS showed higher and significant expression of B-Catenin compared to treatment without FBS ($P<0.05$). While VEGF showed reduced expression when supplemented with 2% FBS, the expression of BFGF supplemented with 2% FBS was slightly increased in both groups, *Nigella sativa* and *Trigona* honey (Figure 3).

One of the important signalling pathway involved in regulation of MSC proliferation and survival is canonical Wnt/β-Catenin signalling (Rodrigues, Griffith & Wells 2010). In the presence of Wnt signalling, activated β-Catenin will be translocated into the nucleus, binds to Lef/Tcf transcription factors, and thus activating the target genes (Reya & Clevers 2005). C-Myc is an oncogene that plays important role in wide-range of cellular processes including cell growth, cell apoptosis and cell differentiation. From the result, it showed that the expression of C-Myc is increased as the expression of β-Catenin increased. This finding confirmed previous studies that identified C-Myc as one of the Wnt/ β-Catenin target gene involved in stimulating cell proliferation (He et al. 1998; Li et al. 2012). Besides that, the expression of β-Catenin supplemented with 2% FBS was higher than treatment without FBS in both, *Nigella sativa* and *Trigona* honey treated cells. FBS is consisted of growth factors and adhesion factors which is commonly used in cell culture media (Díez et al. 2015). It proved that minimal concentration of FBS co-stimulate the BMMSC proliferation and serum deprivation might slow down the cell proliferation (Shin et al. 2008). *Trigona* honey demonstrated better proliferative effect than *Nigella*







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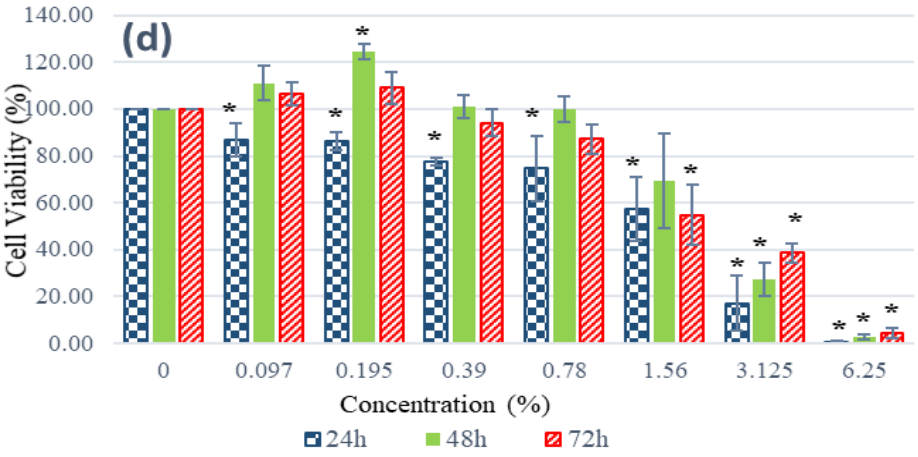


*indicates significant difference at $p < 0.05$ using Mann-Whitney test when compared to control

FIGURE 1. The proliferative effect of (a) *Nigella sativa* without FBS, (b) *Nigella sativa* supplemented with 2% FBS, (c) *Trigona* honey without FBS and (d) Trigona honey supplemented with 2% FBS on BMMSC

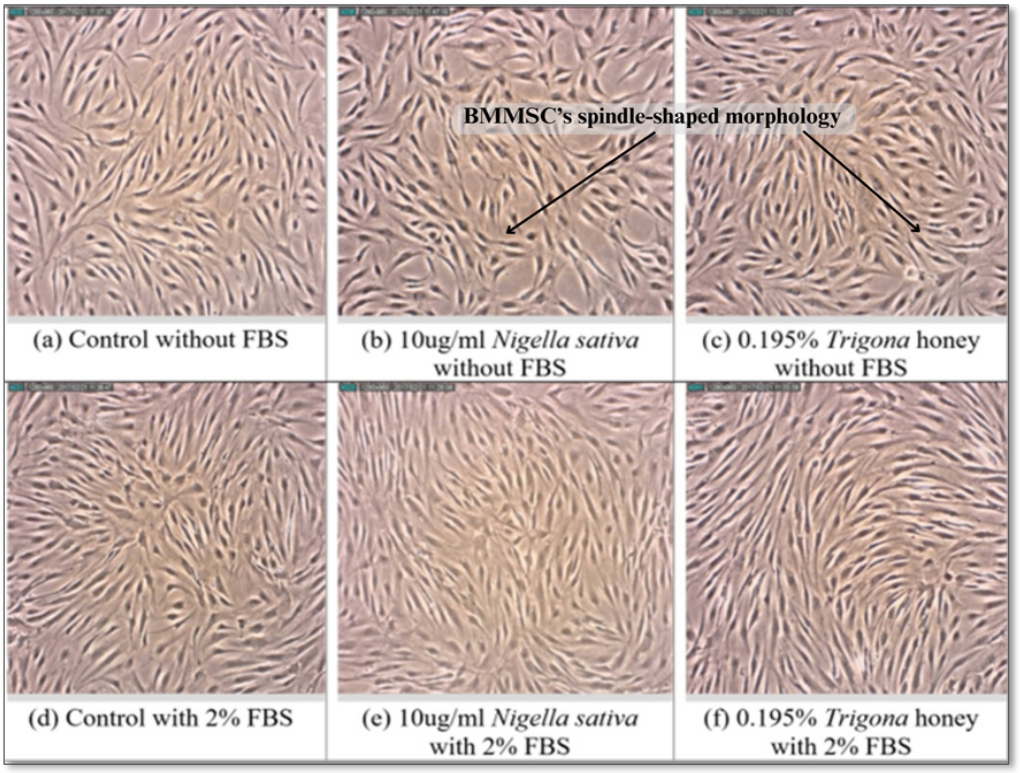
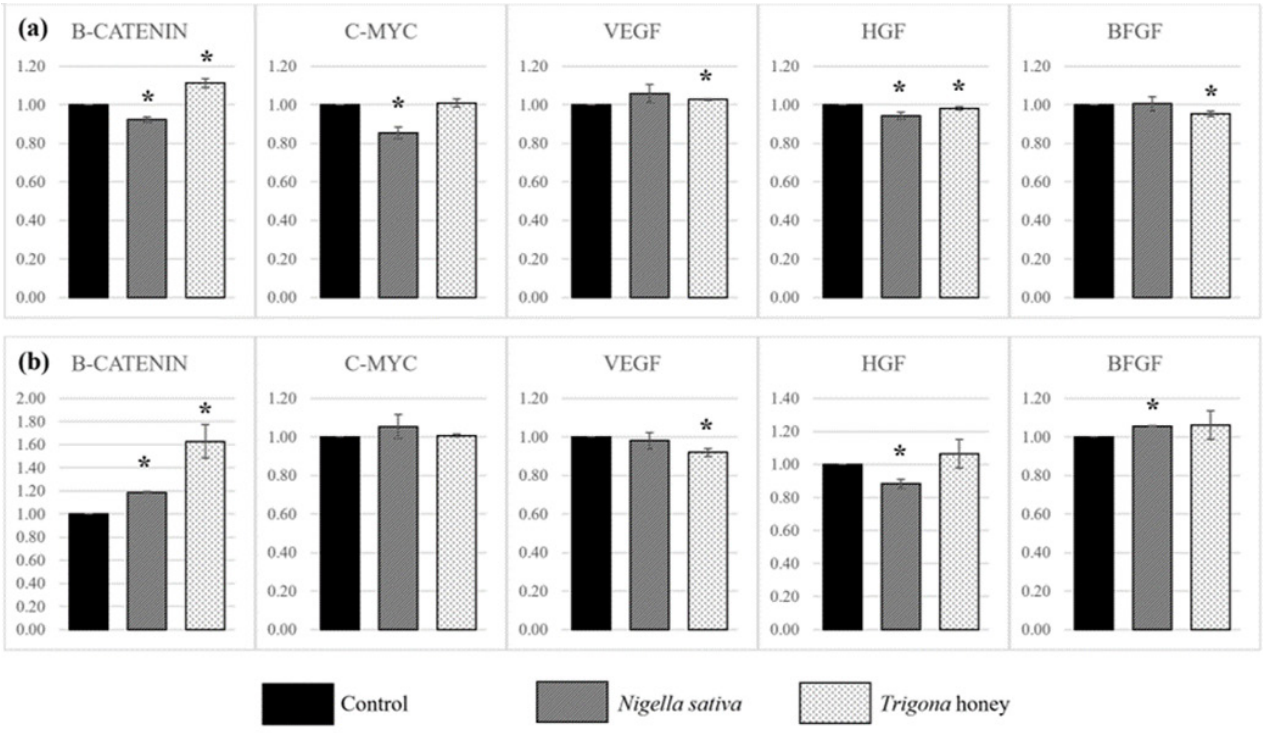


FIGURE 2. Morphology of control and treatment groups after 48 h treatment (100×)

TABLE 2. Surface marker expression of BMMSC. Data are presented as the mean ± standard error of mean (SEM)

Group	Antigen		
	CD73	CD90	CD105
Control 10% FBS	87.4 ± 0.81	91.6 ± 1.7	82.4 ± 0.2
Control No FBS	81.5 ± 0.09	88.3 ± 0.9	84.1 ± 1.5
No FBS *Nigella sativa*	87.3 ± 0.98	92.7 ± 0.1	84.2 ± 1.1
No FBS *Trigona*	78.2 ± 0.92	90.5 ± 2.4	77.3 ± 1.2
Control 2% FBS	83.3 ± 0.69	87.4 ± 0.1	85.6 ± 0.8
2% FBS *Nigella sativa*	86.1 ± 1.13	91.3 ± 1.6	81.6 ± 1.3
2% FBS *Trigona*	82.7 ± 2.48	88.5 ± 2.1	87.9 ± 2.0

Mann-Whitney test, no significant difference at $p > \alpha$ (0.008)



*indicates significant difference at $p < 0.05$ using Mann-Whitney test

FIGURE 3. Relative expression ratio (R) of proliferation-related genes in BMMSC after treatment of *Nigella sativa* and *Trigona* honey (a) without FBS and (b) with 2% FBS

sativa as it still enhanced the BMMSC proliferation in the treatment without FBS. Other than Wnt/β-Catenin signalling, growth factor signalling also plays crucial role in MSC proliferation through upregulation of VEGF, HGF, and bFGF (Nakamura et al. 2011; Rodrigues, Griffith & Wells 2010; Shibuya 2013). Overall, treatment of *Nigella sativa* and *Trigona* honey enhanced the BMMSC proliferation via VEGF in the absence of FBS but signalling through bFGF was observed in the presence of FBS. However, further test is needed to verify the possible mechanisms involved (Table 3).

APOPTOSIS-ASSOCIATED GENES

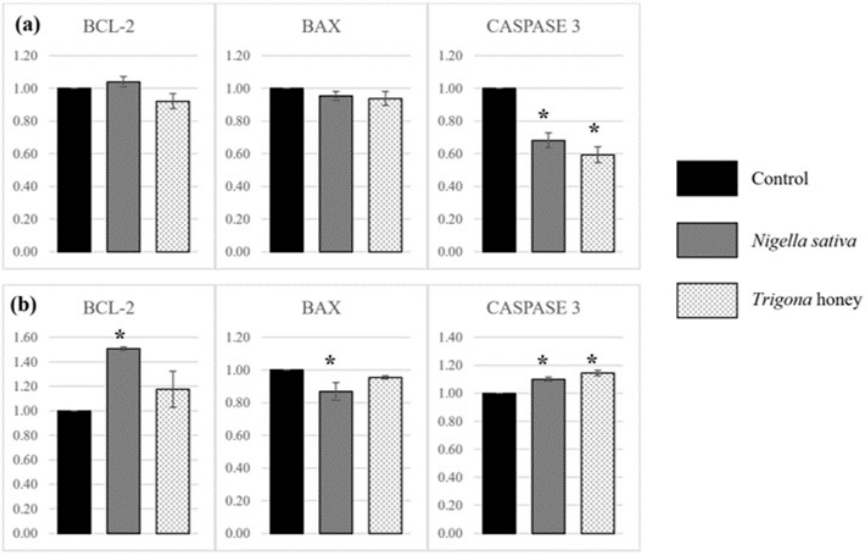
No induction of apoptosis was seen in treatment without FBS as the expression of apoptotic genes, BAX was lower in all groups compared to control (Figure 4). However, Caspase 3 was slightly increased in the treatment with 2% FBS of *Nigella sativa* (1.10) and *Trigona* honey (1.15). BAX and Caspase 3 are pro-apoptotic effectors which involved in cell apoptosis processes (Shalini et al. 2015). Meanwhile, Caspase 3 was slightly increased in groups of *Nigella sativa* and *Trigona* honey supplemented with 2% FBS. Simultaneously, BCL-2 expression also increased as this gene encodes pro-survival protein which blocks the apoptotic death of some cells (Czabotar et al. 2014).

TABLE 3. List of reference genes and target genes. The primer sequences were adapted from previous studies

Genes		Primer sequences (5'-3')	Literature review
TBP	F	TTC GGA GAG TTC TGG GAT TG	Kang et al. (2015)
	R	GGA TTA TAT TCG GCG TTT CG	
β-catenin	F	GCTGATTTGATGGAGTTGGACATGG	Liu and Zhou (2013)
	R	GCCAAACGCTGGACATTAGTGG	
C-Myc	F	AATGAAAAGGCCCCCAAGGTAGTTATCC	Ge et al. (2015)
	R	GTCGTTTCCGCAACAAGTCCTCTTC	
bFGF	F	CCG TTA CCT GGC TAT GAA GG	Tan et al. (2016)
	R	ACT GCC CAG TTC GTT TCA GT	
VEGF	F	CCCACTGAGGAGTCCAACAT	Hayati et al. (2011)
	R	AAATGCTTTCTCCGCTCTGA	
HGF	F	CTGGTTCCCCTTCAATAGCA	Hayati et al. (2011)
	R	CTCCAGGGCTGACATTTGAT	
BCL-2	F	TCCCTCGCTGCACAAATACTC	Minutolo et al. (2012)
	R	ACGACCCGATGGCCATAGA	
BAX	F	TGGAGCTGCAGAGGATGATTG	Xu et al. (2008)
	R	GAAGTTGCCGTCAGAAAACATG	
Caspase 3	F	GCA GCA AAC CTC AGG GAA AC	Zhuo et al. (2009)
	R	TGT CGG CAT ACT GTT TCA GCA	
p16	F	CAACGCACCGAATAGTTACG	Knösel et al. (2014)
	R	CTGCCCATCATCATGACCTGG	
p21	F	TGGAGACTCTCAGGGTCGAAA	Al-Haj, Blackshear, and Khabar (2012)
	R	GGCGTTTGGAGTGGTAGAAATC	

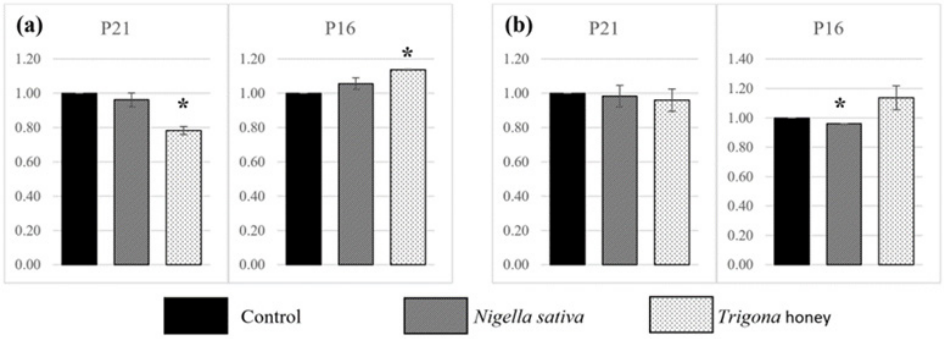
SENESCENCE-ASSOCIATED GENES

Relative expression of p21 was lower in all treatment groups contrary to p16 expression which was increased in all treatment groups except for *Nigella sativa* with 2% FBS (Figure 5). Cell senescence was first introduced by Hayflick and Moorhead (1961) to describe irreversible growth arrest phenomenon where the cells stop dividing after serial cell culture passage. p16 and p21 are cyclin dependent kinase inhibitor which bind to cyclin D complexes and cause cell cycle arrest (Karimian, Ahmadi & Yousefi 2016; Rayess, Wang & Srivatsan 2012). From the result, p21 expression in all groups was lower compared to control which suggest low incidence of cells undergo cell senescence. However, p16 was slightly increased in all treatments. Recently, several studies had found that Wnt/β-Catenin signalling was upregulated in cell senescence of haematopoietic stem cell and MSC (Scheller et al. 2006; Zhang, Wang & Tan 2011). The present finding demonstrated a similar pattern of p16 and β-Catenin relative expression. Hence, it is hypothesized that the elevation of Wnt/β-Catenin could possibly induce the BMMSC senescence through p16 pathway.



*indicates significant difference at p<0.05 using Mann-Whitney test

FIGURE 4. Relative expression ratio (R) of apoptosis-associated genes in BMMSC after treatment of *Nigella sativa* and *Trigona* honey (a) without FBS and (b) with 2% FBS



*indicates significant difference at p<0.05 using Mann-Whitney test

FIGURE 5. Relative expression ratio (R) of senescence-associated genes in BMMSC after treatment of *Nigella sativa* and *Trigona* honey (a) without FBS and (b) with 2% FBS

CONCLUSION

A favourable increased of BMMSC proliferation was observed in all treatments. The proliferative effect was found to be concentration-dependent and time-dependent. Treatment with *Trigona* honey exhibited better proliferative effect compared to *Nigella sativa*. Furthermore, treatment with both extracts did not alter the BMMSC stemness properties and showed low BMMSC apoptosis. Lastly, there was low incidence of cell senescence through p16 pathway as the expression was slightly increased when Wnt/β-Catenin signalling elevated. As a conclusion, it is suggested that *Nigella sativa* and *Trigona* honey possess promising effect on stem cell proliferation.

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*Corresponding author; email: nurfariha@usim.edu.my