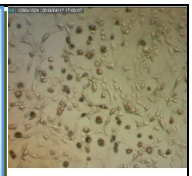
JOBIMB, 2025, Vol 13, No 1, 95-99
https://doi.org/10.54987/jobimb.v13i1.1084

JOURNAL OF BIOCHEMISTRY, MICROBIOLOGY AND BIOTECHNOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/JOBIMB/index



Effects of Malaysian *Kelulut* Honey on Osteoblastic Proliferation of MC3T3-E1 Cells

Nur Najihah Ahmad Puat[1]*, Yong Yoke Keong[1] and Nizar Abd Manan[1]*

[1]Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

*Corresponding author:
Nizar Abd Manan and Nur Najihah Ahmad Puat
Department of Human Anatomy,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 UPM Serdang,
Selangor,
Malaysia.
Email: nurnajihah.ahmadpuat@gmail.com, nizar@upm.edu.my

History

Received: 29th March 2025
Received in revised form: 1st May 2025
Accepted: 30th July 2025

Keywords

Malaysian Kelulut Honey
Osteoblasts
MC3T3-E1 Cells
Proliferation
Cell viability

Abstract

Malaysian *Kelulut* Honey (MKH) is a well-recognized Malaysian honey and has gained widespread popularity due to its ability to promote health. Numerous scientific studies have been conducted to prove the health benefits of MKH; however, its bone-protective effects have not yet been well-studied. Osteoblasts are vital cells for bone formation; thus, it is important to study the effect of MKH on osteoblasts for a better understanding of bone formation. This study was conducted to evaluate the effects of MKH on the proliferation of bone-forming cells using MC3T3-E1 cells. The cytotoxicity and proliferation of MKH on MC3T3-E1 cells were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and the bromodeoxyuridine (BrdU) assay. The cells were treated with MKH at various concentrations ranging from 0.001% v/v to 10.0% v/v for 24, 48, and 72 hours. The results demonstrated that MKH at 0.01% v/v significantly enhanced proliferation by 131.65% ($p<0.001$) after 24 hours compared to the negative control. However, cell viability and proliferation decreased in a dose-dependent manner at concentrations ≥0.05% v/v MKH, with 10.0% v/v MKH showing notable reduction in proliferation by over 40% ($p<0.001$) across all time points. Besides that, MKH concentrations below 0.1% v/v were non-cytotoxic and promoted osteoblast growth, indicating a limited effective range. These results suggest that MKH at low concentrations enhances osteoblast proliferation, supporting its potential as a natural agent for bone health.

INTRODUCTION

Osteoporosis is a metabolic bone disease that primarily affects the elderly. It's generally characterized by reduced bone density, malfunctioning bone tissues, and a higher risk of fracture. While osteoporosis affects both men and women, women are more likely to experience severe complications [1]. This disease is known as the "silent disease" because most people are unaware they have it until they break a bone from a slight fall or bump that would not otherwise cause such a severe injury. Osteoporosis affects nearly 200 million people worldwide, making it one of the most serious current public health problems. According to the International Osteoporosis Foundation (IOF), one in every three women over the age of 50 and one in five men will suffer an osteoporotic fracture during their lifetime. On the other hand, osteoblasts, which are specialized cells in bone formation, may have the potential to improve osteoporosis since a reduction in osteoblasts increases bone susceptibility to fracture. Thus, osteoblasts play a pivotal role in the study of osteoporosis, as these cells synthesize and deposit the organic matrix of bone, which is primarily composed of collagen fibers [2,3]. These cells also regulate the process of mineralization by controlling the deposition of calcium and other minerals onto this matrix. In normal bone remodeling, osteoblasts are essential for building and repairing bone tissue. Bone is a dynamic tissue that undergoes continuous remodeling, involving both bone resorption (breakdown) and bone formation. Moreover, osteoblasts work in tandem with osteoclasts, which are responsible for bone resorption [3–5]. Hence, reduced bone formation and increased bone resorption may lead to osteoporosis.

Teriparatide, denosumab, parathyroid hormone, cathepsin K inhibitors, hormone replacement therapy, and selective estrogen receptor modulators are all currently used to treat osteoporosis [6]. However, most of the synthetic therapeutic agents cause undesirable side effects among postmenopausal women, such as cancer, stroke, and coronary heart disease [7]. Due to these

reasons, an alternative agent with therapeutic potential and few adverse effects is needed. Honey is known as a traditional remedy in the treatment of various types of diseases. Honey contains various biological properties, including antimicrobial, wound and sunburn healing, antioxidant, antiviral, anti-inflammatory, antiparasitic, anti-diabetic, anti-mutagenic, and antitumor activities [8,9]. The murine preosteoblast cell line (MC3T3-E1), obtained from mice, is frequently used as an *in vitro* experimental model to assess the ability of osteogenic differentiation and proliferation. Osteoblasts have the ability to express extracellular matrix proteins, including osteocalcin, alkaline phosphatase (ALP), and other bone matrix proteins, throughout differentiation [10]. Despite extensive research highlighting the benefits of Malaysian stingless bee (*Kelulut*) honey (MKH), its impact on MC3T3-E1 cell proliferation remains unexplored. Therefore, the present study was conducted with the aim to explore the optimal concentration of MKH for its cytotoxicity and proliferative effects on MC3T3-E1 cells using MTT and BrdU assays. Thus, these results provide an initial insight into the effects of MKH on osteoblasts and bone diseases related to osteoblasts.

MATERIALS AND METHODS

Cell culture

MC3T3-E1 cells (cell no. RCB1126) were acquired from Riken, Japan, and cultured in a complete growth culture medium consisting of alpha-modified minimum essential media eagle (αMEM) (Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS) (Tico Europe, Netherlands), 1% penicillin-streptomycin (Nacalai Tesque, Japan), and amphotericin B (Sigma Aldrich, USA). Cells were maintained at 37°C in 5% CO_2. For all experiments, cells were utilized at 80%-90% confluency and within passages six to ten. The culturing of MC3T3-E1 cells followed the standard protocol stated by Wang et al. [11].

Preparation of Malaysian *Kelulut* Honey

MKH was obtained from local stingless bee apiarists. Prior to cell treatment, MKH was prepared at a final concentration of 20% v/v using culture media, based on previous honey preparation methods with slight modifications [12,13]. Various concentrations of MKH were prepared from the stock solution and sterilized with a 0.2 µm syringe filter (Bioflow, Malaysia). Subsequently, MC3T3-E1 cells were treated with different MKH concentrations ranging from 0.01% v/v up to 10% v/v.

Cell Viability and Cytotoxicity via MTT assay

Cell viability and cytotoxicity of MC3T3-E1 cells were assessed via MTT assay based on an established protocol with minor modifications [14]. MC3T3-E1 cells were cultured in a 96-well plate at a density of 1.0×10^4 cells/well and treated with various concentrations of MKH (0.01%, 0.05%, 0.1%, 0.5%, 1.0%, 5%, and 10%). Untreated cells served as the negative control. The cells were incubated at 37°C in a humidified incubator for 24, 48, and 72 hours. Following each incubation period, 20µL of MTT solution was added to each well before further incubation for 4 hours. The absorbance of the formazan was measured using a spectrophotometer microplate reader (SpectraMax Plus 384, Molecular Device, USA) at a wavelength of 570 nm and the measurements were performed 24, 48, and 72 hours after treatment.

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treatment (570 nm)}}{\text{Absorbance of control (570 nm)}} \text{ x } 100$$

Cell Proliferation via BrdU assay

MC3T3-E1 cell proliferation was assessed using the Bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit (Millipore©, Merck, Germany) following the manufacturer's instruction, based on the original method which involves the incorporation of BrdU during DNA synthesis for the detection of proliferating cells [15]. Briefly, the cells were cultured in a 96-well plate at $2.0X10^5$ cells/well and treated with multiple concentrations of test substances for 24, 48, and 72 hours, respectively. The untreated cells served as the negative control. BrdU solution was added to the appropriate wells for 2 hours before the end of the incubation period. The cells were fixed and the DNA denatured when the culture media was removed. Next, in order to quantify the amount of integrated BrdU, anti-BrdU antibody was added. The microplate scanning spectrophotometer was used to measure the absorbance of each well at 450 nm.

$$\text{Cell Proliferation (\%)} = \frac{\text{Absorbance of treatment (450nm)}}{\text{Absorbance of control (450nm)}} \text{ x } 100$$

Statistical Analysis

The results were presented as means ± standard error mean (SEM), and statistical significance was assessed using one-way analysis of variance (ANOVA) with post-hoc Dunnett tests for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. The analysis was carried out using the Statistical Package for the Social Sciences (SPSS), specifically IBM SPSS Statistics version 20 with all procedures performed in triplicate (n = 3).

RESULTS

Cell cytotoxicity and viability

The results of MKH-treated groups were also compared with the negative group (MC3T3-E1 cells without treatment). The percentage of viable cells was assessed at different time points, namely at 24, 48, and 72 hours. Based on **Fig. 1**, there was no significant difference shown at 0.01% v/v MKH, although the cell viability was increased when compared to the negative control. The viability of MC3T3-E1 cells decreased as the MKH concentrations increased. Significant differences were observed in decreasing viability of MC3T3-E1 cells treated with MKH at concentrations of 0.5% v/v ($p<0.05$), 1.0% v/v, 5.0% v/v and 10.0% v/v ($p<0.001$) when compared to the negative control at 24, 48 and 72 hours. Thus, the study showed that MKH at concentrations lower than 0.1% v/v were not cytotoxic to MC3T3-E1 cells.

Cell Proliferation via BrdU assay

After 24 hours of treatment, the proliferation of MC3T3-E1 cells treated with MKH concentrations from 0.01% v/v to 0.5% v/v increased compared to the negative control group (**Fig. 2**). However, only the proliferation of MC3T3-E1 cells treated with MKH at the concentration of 0.01% v/v increased significantly ($p<0.001$) against the negative control, up to 131.65%. However, there was no significant difference against the negative control shown on the lower MKH concentrations after 48 and 72 hours of treatment. Apart from that, cell proliferation started to decrease when treated with 1.0% v/v to 10% v/v MKH as the MKH concentration increased. At the highest MKH concentration (10% v/v), a significant decrease in MC3T3-E1 cell proliferation ($p<0.001$) was observed against the negative control across all time points (24, 48, and 72 hours).

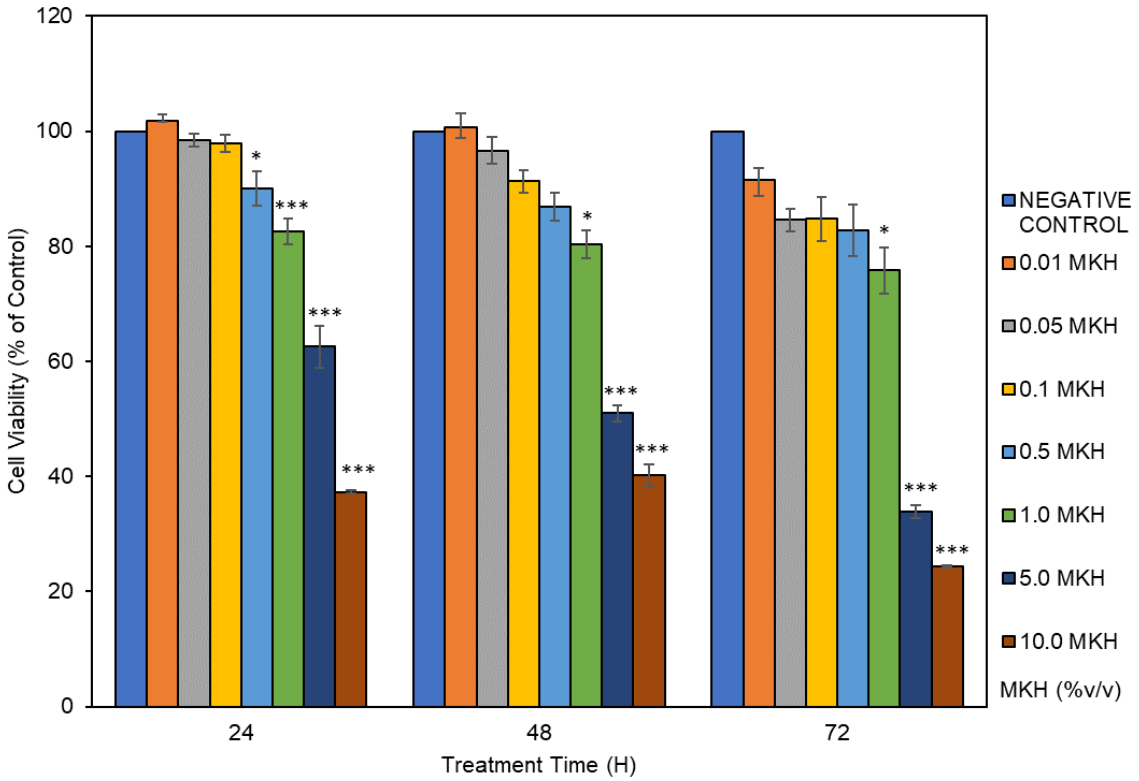


Fig. 1. Effect of MKH on the viability of MC3T3-E1 cells at different time points (24, 48, and 72 hours). Data are expressed as mean ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control for 24, 48, and 72 hours.

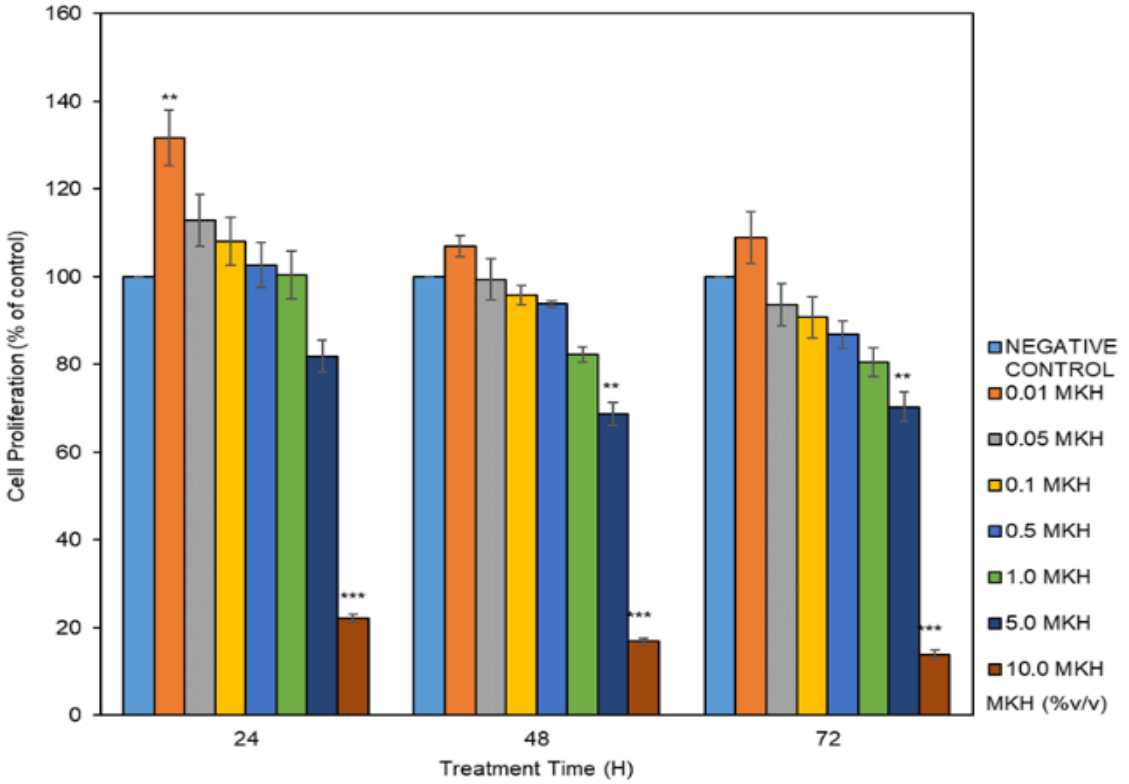


Fig. 2. Effect of MKH on proliferation of MC3T3-E1 cells at different time points (24, 48, and 72 hours). Data are expressed as mean ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control for 24, 48, and 72 hours.

DISCUSSION

The aim of this study was to determine the impact of MKH concentration on the osteogenic activity of MKH on MC3T3-E1 cells, and thus, assess MKH's potential to serve as a bone nourishment. The results showed that MKH had a significant effect on osteoblasts, specifically in terms of cell proliferation, at low concentrations of MKH compared to the negative control after 24 hours. Cell viability refers to the proportion of live and dead cells in a population, which should parallel cell proliferation in a non-cytotoxic culture system. The viability results obtained in this study were similar to those of other studies involving honey conducted on different types of cells. Furthermore, the results showed a reduction in viability in various cell lines, including keratinocytes [16], lymphoblastoid cells [17,18], and RAW 264.7 murine macrophage cell lines [18] in a dose-dependent manner when treated with increasing concentrations of MKH.

While high concentrations of MKH exhibited cytotoxic effects on MC3T3-E1 cells, the underlying mechanism remains speculative. We postulate that this effect may stem from increased production of reactive oxygen species (ROS) and elevated oxidative stress markers, a hypothesis supported by similar findings in studies of acidic and hyperosmolar natural extracts [13]. The acidic nature of honey or its hyperosmolarity is known to contribute to its excellent antimicrobial properties. However, the same characteristics could also contribute to its toxicity at high concentrations. Given that honey's pH typically falls within the range of 3.5 to 5.5, these properties could potentially influence osteoblast proliferation in culture [16,19]. Similar to natural plant extracts, high concentrations of honey could also be cytotoxic [20,21]. MKH, when present at concentrations above 1.0% v/v, was observed to inhibit osteoblast proliferation. It is hypothesized that the acidic characteristics of MKH, coupled with its high water activity, lower pH (3.04), and increased free acidity (347.5 meq/kg), may collectively hinder osteoblast development [22].

An optimal pH range for most cells in culture is between 7.2 and 7.4. Therefore, any alteration of pH in the cell culture medium can impede the proliferation of the cells. Even though the pH alteration of cell culture medium caused by acidic honey may be buffered [16], it can unfortunately still contribute to the inhibition of osteoblast proliferation (18). Consequently, lower MKH concentrations, specifically ranging from 0.0025% v/v to 0.1% v/v, were chosen for this experiment, as these concentrations are deemed safe for assessing the differentiation and mineralization of MC3T3-E1 cells. While this study demonstrated that MKH enhances osteoblastic proliferation at low concentrations, proliferation alone does not confirm osteogenic potential.

For effective bone formation, osteoblasts must differentiate and express bone-specific markers. Thus, future studies should comprehensively explore MKH's osteogenic potential through differentiation assays, including alkaline phosphatase (ALP) activity, mineralization assessment via Alizarin Red staining, osteocalcin quantification and gene expression analysis of RUNX2, to fully determine the impact of MKH on osteoblast maturation. This approach aligns with findings by Abdelaziz et al. [23], who observed upregulation of RUNX2, COL1A1, and osteocalcin in MG-63 cells cultured on flaxseed extract-loaded nanofiber scaffolds, and by Xing et al. [24], who reported increased levels of ALP, RUNX2, and osteocalcin during mineralization in MC3T3-E1 cells. Furthermore, while MKH demonstrated cytotoxic effects at higher concentrations, this study did not directly investigate oxidative stress or mitochondrial dysfunction. It is postulated that the acidic or hyperosmolar properties of MKH may contribute to increased ROS generation and mitochondrial impairment, a hypothesis supported by previous findings. Jaara and Torres [25] identified mitochondrial ROS as a key driver of inflammasome activation and cell death. Therefore, future studies should verify the involvement of these pathways by including targeted assays such as DCFH-DA for ROS detection, JC-1 staining for mitochondrial membrane potential, and ATP quantification. Additionally, *in vivo* validation using established osteoporosis models, such as ovariectomized rodents, is essential to determine the systemic effects of MKH on bone architecture, density, and mechanical integrity. These studies will contribute to a more comprehensive understanding of MKH's therapeutic potential for bone health and its possible application in osteoporosis management. Based on the outcomes of this study, it can be concluded that MKH at low concentrations increased the proliferation of MC3T3-E1 cells. Therefore, MKH demonstrates potential to assist in bone formation.

LIST OF ABBREVIATIONS

αMEM - alpha modified minimum essential media eagle
ALP - Alkaline Phosphatase
ANOVA – one-way analysis of variance
ATP - Adenosine Triphosphate
BrdU assay - bromodeoxyuridine assay
DCFH-DA – 2′,7′-Dichlorofluorescin Diacetate
FBS – Fetal Bovine Serum
IOF - International Osteoporosis Foundation
JC-1 - 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
MKH – Malaysian *Kelulut* Honey
MTT assay - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay
NO - nitric oxide
ROS - reactive oxygen species

ACKNOWLEDGEMENT

This research was funded by the Ministry of Higher Education Malaysia, under the Fundamental Research Grant Scheme (FRGS/1/2017/SKK06/UPM/02/18). The authors would like to extend their gratitude to the staff of the Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) for their invaluable technical support.

REFERENCES

1. Thu HE, Mohamed IN, Hussain Z, Shuid AN. Exploring molecular mechanism of bone-forming capacity of Eurycoma longifolia: Evidence of enhanced expression of bone-related biomarkers. J Ayurveda Integr Med. 2018;9(4):272–80.
2. Florencio-Silva R, da Silva Sasso GR, Sasso-Cerri E, Jesus Simões M, Sérgio Cerri P. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. Immuno-Anal Biol Spéc. 2015;2015(6):17.
3. Kenkre JS, Bassett JHD. The bone remodelling cycle. Ann Clin Biochem. 2018;55(3):308–27.
4. Crous A, Abrahamse H. The Signalling Effects of Photobiomodulation on Osteoblast Proliferation, Maturation and Differentiation: A Review. Stem Cell Rev Rep. 2021;1570–89.
5. Ralston SH. Bone structure and metabolism. Med U K. 2017;45(9):560–4.
6. Tu KN, Lie JD, Wan CKV, Cameron M, Austel AG, Nguyen JK, et al. Osteoporosis: A review of treatment options. P T. 2018;43(2):92–104.

7. Skjødt MK, Frost M, Abrahamsen B. Side effects of drugs for osteoporosis and metastatic bone disease. Br J Clin Pharmacol. 2019;85(6):1063–71.
8. Oryan A, Alemzadeh E, Moshiri A. Biological properties and therapeutic activities of honey in wound healing: A narrative review and meta-analysis. J Tissue Viability. 2016;25(2):98–118.
9. Rao PV, Krishnan KT, Salleh N, Gan SH. Biological and therapeutic effects of honey produced by honey bees and stingless bees: A comparative review. Braz J Pharmacogn. 2016;26(5):657–64.
10. Guan M, Pan D, Zhang M, Leng X, Yao B. The Aqueous Extract of Eucommia Leaves Promotes Proliferation, Differentiation, and Mineralization of Osteoblast-Like MC3T3-E1 Cells. Evid Based Complement Alternat Med. 2021;2021.
11. Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH, Franceschi RT. Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res. 1999;14(6):893–903.
12. Devasvaran K, Tan JJ, Ng CT, Fong LY, Yong YK. Malaysian Tualang Honey Inhibits Hydrogen Peroxide-Induced Endothelial Hyperpermeability. Oxid Med Cell Longev. 2019;2019.
13. Khalid AFBA, Ng CT, Fong LY, Tan JJ, Yakop FB, Kamaruddin NAB, et al. Malaysian Tualang Honey Suppresses the Angiogenic Events in Endothelial Cells Induced by Vascular Endothelial Growth Factor. Sains Malays. 2022 Mar 1;51(3):815–22.
14. Mosmann TR. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65 1-2:55–63.
15. Gratzner H G. Monoclonal Antibody to 5-Bromo-and 5-Iododeoxyuridine: A New Reagent for Detection of DNA Replication [Internet]. 1982 p. 474–5. (Science). Available from: 10.1126/science.7123245
16. Nordin A, Chowdhury SR, Saim AB, Idrus RBH. Effect of kelulut honey on the cellular dynamics of tgfβ-induced epithelial to mesenchymal transition in primary human keratinocytes. Int J Environ Res Public Health. 2020;17(9).
17. Hazirah H, Yasmin AM, Abdul Karim N. Antioxidant Properties of Stingless Bee Honey and Its Effect on the Viability of Lymphoblastoid Cell Line. Med Health. 2019;14(1):91–105.
18. Ooi TC, Yaacob M, Rajab NF, Shahar S, Sharif R. The stingless bee honey protects against hydrogen peroxide-induced oxidative damage and lipopolysaccharide-induced inflammation in vitro. Saudi J Biol Sci. 2021;28(5):2987–94.
19. Yu LX, Taib H, Berahim Z, Ahmad A, Zainuddin SLA. The effect of Tualang honey on human periodontal ligament fibroblast proliferation and alkaline phosphatase level. Sains Malays. 2015;44(7):1021–5.
20. Kannan TP, Ali AQ, Abdullah SF, Ahmad A. Evaluation of Tualang honey as a supplement to fetal bovine serum in cell culture. Food Chem Toxicol. 2009;47(7):1696–702.
21. Yusof AM, Abd Ghafar N, Kamarudin TA, Hui CK, Yusof YAM. Gelam honey potentiates ex vivo corneal keratocytes proliferation with desirable phenotype expression. BMC Complement Altern Med. 2016;16(1):1–9.
22. Zhang Z, Lai Q, Li Y, Xu C, Tang X, Ci J, et al. Acidic pH environment induces autophagy in osteoblasts. Sci Rep [Internet]. 2017 Apr 6 [cited 2025 July 16];7(1). Available from: https://www.nature.com/articles/srep46161
23. Abdelaziz AG, Nageh H, Abdalla MS, Abdo SM, Amer AA, Loutfy SA, et al. Development of polyvinyl alcohol nanofiber scaffolds loaded with flaxseed extract for bone regeneration: phytochemicals, cell proliferation, adhesion, and osteogenic gene expression. Front Chem. 2024;12.
24. Xing L, Li Y, Li W, Liu R, Geng Y, Ma W, et al. Expression of RUNX2/LAPTM5 in the Induction of MC3T3-e1 Mineralization and Its Possible Relationship with Autophagy. Tissue Eng Regen Med. 2022 Dec 1;19(6):1223–35.
25. Jaara HS, Torres S. Mitochondrial ROS, a trigger for mitochondrial dysfunction and inflammasome activation and a therapeutic target in liver diseases. Explor Dig Dis. 2024 Dec 10;3(6):474–503.