IN VITRO OSTEOGENIC ACTIVITY OF MALAYSIAN KELULUT HONEY ON MURINE PREOSTEOBLASTS MC3T3-E1 CELLS

By

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Malaysian Kelulut Honey (MKH) is a well-recognised Malaysian honey and has gained widespread popularity due to its ability to promote health. Numerous scientific studies have been done to prove the health benefits of MKH; however, there is no evidence on the bone protective effects of MKH in osteogenic condition studied yet. Thus, this study aimed to explore the effect of MKH on bone by using murine preosteoblast cell line (MC3T3-E1) with an intention to establish MKH as one of the natural products which may promote osteoblast formation. The MC3T3-E1 were cultured and treated with honey at various concentration (0.001% v/v to 10.0% v/v) in a 96-well plate and incubated at 37°C with 5% CO_2. Cytotoxicity test of MKH on the viability and proliferation of MC3T3-E1 cells were assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and bromodeoxyuridine (BrdU) assay up to 72 hours. The concentrations of MKH in the range of 0.01% v/v to 0.1% v/v showed no cytotoxic effect on MC3T3-E1 cells. Besides cell growth, the ability of MKH to promote osteoblastic differentiation was also investigated. The αMEM supplemented with 100 nM dexamethasone, 0.05 µM ascorbate-2-phosphate, and 10 mM β-glycerophosphate was used as negative control. The result of osteoblastogenesis showed that ALP activity ($p<0.05$) and deposition of collagen ($p<0.001$) increased significantly when compared to their negative control respectively on day 21. However, there were increased in other aspects of osteoblastic differentiation, such as colony formation, calcium and inorganic-phosphate deposition, concentration of osteocalcin (OCN) and osteoprotegerin (OPG) and bone-specific marker mRNAs expressions on day 21 without any significant difference compared to the negative control. Hence, the role of MKH on osteoblasts in osteogenic activity requires further investigation.

Keywords: honey, kelulut, osteoporosis, osteoblast, stingless bee

SGD: GOAL 4: Quality Education

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

AKTIVITI OSTEOGENIK SECARA *IN VITRO* MADU KELULUT MALAYSIA TERHADAP SEL SELANJAR PRAOSTOBLAST MENCIT MC3T3-E1

Oleh

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Madu Kelulut Malaysia (MKH) adalah merupakan salah satu madu Malaysia yang terkenal dan mendapat sambutan meluas kerana keupayaannya untuk meningkatkan tahap kesihatan. Banyak kajian saintifik telah dilakukan untuk membuktikan manfaat MKH kepada kesihatan; namun, kesan MKH terhadap perlindungan tulang masih belum dikaji dengan mendalam. Oleh itu, kajian ini bertujuan untuk meneroka kesan MKH kepada tulang dengan menggunakan sel selenjar praosteoblast mencit (MC3T3-E1) dengan tujuan untuk menjadikan MKH sebagai salah satu produk semula jadi yang boleh menggalakakan pertumbuhan osteoblast. MC3T3-E1 dikultur dan dirawat dengan pelbagai kepekatan berbeza (0.001% v/v to 10.0% v/v) pada plat 96-takung dan dieram pada 37°C dengan 5% CO_2. Ujian sitotoksisiti MKH terhadap viability dan proliferasi sel MC3T3-E1 telah dinilai maisng-masing menggunakan asai 3-(4,5-dimetitiazol-2-yl)-2,5-difeniltetrazolium bromida (MTT) dan asai bromodeoksiuridin (BrdU) sehingga 72 jam. Kepekatan MKH dalam julat 0.01% v/v hingga 0.1% v/v tidak menunjukkan kesan sitotoksisiti terhadap sel MC3T3-E1. Selain pertumbuhan sel, keupayaan MKH untuk menggalakkan pembezaan osteoblastik juga telah dikaji pada hari ke 14 dan 20 kajian. αMEM ditambah dengan 100 nM deksametason, 0.05 µM askorbat-2-fosfat, and 10 mM β-gliserofosfat digunakan sebagai kawalan negatif. Keputusan osteoblastogenesis menunjukkan aktiviti ALP Keputusan osteoblastogenesis menunjukkan bahawa aktiviti ALP ($p<0.05$) dan pertumbuhan kolagen ($p<0.001$) meningkat secara signifikan apabila dibandingkan dengan kawalan negatif masing-masing pada hari ke-21. Walau bagaimanapun, terdapat peningkatan dalam asai pembezaan osteoblast yang lain seperti pembentukan koloni, kalsium dan fosfat tidak organik, kepekatan osteocalcin (OCN) dan osteoprotegerin (OPG) dan ekspresi mRNAs penanda tulang pada hari ke-21 tanpa perbezaan yang signifikan berbanding kawalan negatif. Oleh itu, peranan MKH pada osteoblas dalam aktiviti osteogenik memerlukan penyelidikan lanjut.

Kata Kunci: kelulut, lebah tidak bersengat, madu, ostoporosis, ostoblast

SGD: MATLAMAT 4: Pendidikan Berkualiti

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date:12 September 2024

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<	less than
>	more than
+ve	positive
-ve	negative
%	percentage
3D	3 dimension
µg	microgram
µm	micrometre
µL	microlitre
αMEM	Minimum Essential Media Eagle
AACE/ACE	American Association of Clinical Endocrinologists or American College of Endocrinology
ALP	alkaline phosphatase
BLC	bone lining cells
BMD	bone mass density
BMU	Basic Multicellular Unit
BrdU	bromodeoxyuridine
BSP	*bone sialoprotein*
CCM	complete culture media
Cl^-	chloride ion
COLα1	*collagen type 1 alpha-1*
DM	differentiation media
DMSO	dimethyl sulfoxide
ECM	extracellular matrix

ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinases
FDA	Food and Drug Administration
FBS	fetal bovine serum
H	hours
HCl	hydrochloric acid
IOF	International Osteoporosis Foundation
IOM	Institute of Medicine
IU	International unit
nm	nanometre
mL	millimetre
MKH	Malaysian Kelulut Honey
MRSA	methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium
NaOH	sodium hydroxide
OCN	osteocalcin
OD	optical density
OPN	osteoprotegerin
PBS	phosphate buffered saline
pNpp	p-Nitrophenyl Phosphate
pNp	p-Nitrophenol
PTH	parathyroid hormone
ROS	reactive oxygen species
Runx2	*Runt-related transcription factor 2*

TPTD	teriparatide
TMB	tetramethylbenzidine
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Osteoporosis is a metabolic bone disease that affects mostly the elderly. In general, osteoporosis is characterised by a reduction in bone density, malfunctioning bone tissues, and a higher risk of fracture. Osteoporosis affects both men and women, but women are more likely to experience severe osteoporosis complications (Thu et al., 2018). This disease may affect nearly 500 million people worldwide, thus, making it one of the current most serious public health problems. It is estimated 6.3% of men and 21.2% of women over the age of 50 suffered on osteoporosis (Chen et al., 2020).

Osteoporosis is caused by a variety of factors, including genetic and environmental factors (Al Anouti et al., 2019; Baudoin et al., 2002). This disease is known as the "silent disease" because most people are unaware that they have osteoporosis until their bone suffer from a slight fall or bump that would not resulted in such a severe injury (Nguyen, 2017; Tu et al., 2018). Surprisingly, even after breaking a bone, around 80% of patients are still not diagnosed and treated for osteoporosis properly, which is the underlying condition that causes the fracture.

Patients with osteoporosis usually take drugs recommended by their physicians. Teriparatide, denosumab, parathyroid hormone, cathepsin K inhibitors, hormone replacement therapy and selective oestrogen receptor modulators are all currently used to treat osteoporosis (Tu et al., 2018). However, the majority of the synthetic therapeutic agents are related to bad side effects in postmenopausal women, such as cancer, stroke and coronary heart disease (Skjødt et al., 2019; Tu et al., 2018). Given these reasons, an alternative agent with therapeutic potential and fewer adverse effects is needed.

Generally, honey is known as a traditional remedy in the treatment of various types of diseases (Eteraf-oskouei & Najafi, 2013; Nweze et al., 2020; Samarghandian et al., 2017). Honey contains various types of biological properties, including antimicrobial, wound and sunburn healing, antioxidant, antiviral, anti-inflammatory, antiparasitic, anti-diabetic, anti-mutagenic and antitumor activities (Oryan et al., 2016; Rao et al., 2016). Although numerous studies have been conducted on the beneficial effects on honey exhibited by stingless bees, the potential of Malaysian Kelulut Honey (MKH) in the study of the osteogenic activity of preosteoblast cell line (MC3T3-E1) has not been investigated. Hence, this study aims to investigate the osteogenic activity of MKH on MC3T3-E1 cells and thus, uncover the potential of MKH to serve as nourishment for bones.

1.2 Problem statement

Bone is a biological organ that constantly moulds itself. Bone undergoes a life-long remodelling process that includes bone resorption by osteoclasts and bone formation by osteoblasts. Osteoporosis is a major bone metabolic disease that develops with age, especially in women after menopause. The imbalance in bone remodelling, specifically an increase in bone resorption and a decrease in bone formation, triggers osteoporosis (Lai et al., 2014). However, treatment of osteoporosis slowly becomes a burden on society, especially in terms of the cost. Therefore, people prefer to treat osteoporosis by non-pharmacologically instead of going to the expert to undergo the current treatment. Nonetheless, limitations in the knowledge about osteoporosis and the low monthly income associated with osteoporosis due to limited access to healthcare, lack of access to healthy food and supplements, and lack of engagement with medical professionals. Hence, it contribute to the number of patients suffering from osteoporosis, especially in Malaysia (Subramaniam et al., 2019).

In Europe, the number of fragility fractures was expected to rise from 3.5 million in 2010 to 4.5 million in 2025, corresponding to an increase of 28% (Kanis et al., 2020). Besides, in the USA, over 1.5 million fractures per year were attributable to osteoporosis, resulting in direct healthcare costs of 12–18 billion US dollars (Clynes et al., 2020). It is also projected that by 2025, more than 3 million cases of osteoporotic fracture will occur annually, with an estimated cost of 25.3 billion dollars. Hence, improving osteoporosis care and reducing spiralling fracture-related costs have posed worldwide challenges (Kemmak et al., 2020; Li et al., 2021). Furthermore, the use of drug such as bisphosphonates which expensive saw 50% decrease in users from 2008 to 2012 in United State and supported by the study in United Kingdom due to public awareness about devastating side effects such as typical femur and osteonecrosis of the jaw. Particularly, current drugs are welcome but not the full answer for fracture prevention in the ageing population. Hence, it is important to realise that effective drugs reduce fracture rates, but do not fully prevent the occurrence of fractures (Lems & Raterman, 2017).

In this regard, as an alternative way, honey is used as a health supplement daily by most people due to its avaibility. Other than that, honey is not only highly nutritious but it is safe to be consumed by all generations. Beside that, the interest in the use of honey in treating a wide array of diseases rather than only limited to wound healing (Rosli et al,. 2020). However, the studies regarding the effect of honey on bone metabolism is still deficient. Malaysian Kelulut honey (MKH) is a health elixir from stingless bees (from *Trigona* sp.), which has gained interest to be developed as a superfood. It was also claimed to have higher antioxidants than regular honey (Mardhiah et al., 2022). However, the pharmacological action of this honey remains largely unclear, particularly in bone metabolism. Thus, this study can provide us with an understanding of the effects of MKH on bone metabolism and the findings could be beneficial to human health in the near future.

1.3 Objectives of the study

1.3.1 General objective

To investigate the osteogenic potential of MKH on murine preosteoblast (MC3T3-E1) cell lines and its underlying mechanism of action.

1.3.2 Specific objectives

1) To determine the cytotoxicity effect of MKH on the viability of MC3T3-E1 cells.
2) To assess the effect of MKH on the proliferation of MC3T3-E1 cells.
3) To determine the effects of MKH in MC3T3-E1 cells in osteogenic conditions.
4) To determine the effects of MKH on the expression of the bone-specific mRNAs, namely *Runt-related transcription factor 2* (Runx2), *collagen type 1 alpha-1* (COLα1) and *bone sialoprotein* (BSP) in osteogenic conditions.

1.4 Hypotheses

1) MKH does not cause cytotoxicity to MC3T3-E1 cells.
2) MKH increases the proliferation of MC3T3-E1 cells.
3) MKH promotes osteoblastic differentiation of MC3T3-E1 cells.
4) MKH increases the level and activity of the differentiation and mineralisation of MC3T3-E1 cells.
5) MKH induces the expression of the bone-specific mRNAs of MC3T3-E1 cells.

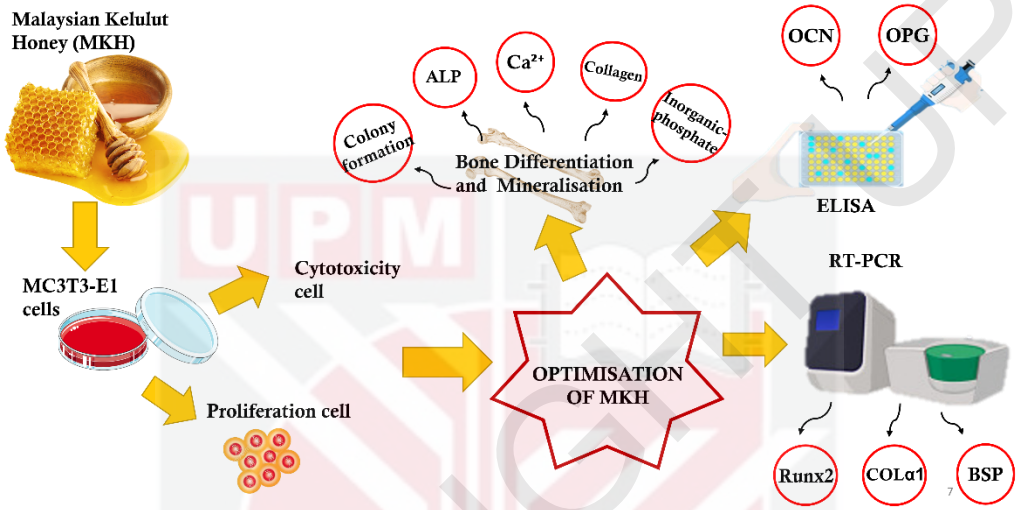
1.5 Significance of the study

Honey is a natural remedy that has been used as traditional medicine and treatment for a long time. In fact, honey has been used as an alternative treatment for wound healing and several other ailments because the elderly believed that honey was both safe and non-harmful to humans. However, the possibility of honey in treating a variety of diseases has not been fully discovered yet.

In Malaysia, the popularity of MKH as a health supplement is increasing; however, scientific studies about MKH are still lacking. As MKH is widely available in the Malaysian market, any health benefit of MKH that is proven scientifically will boost sales and generate income for the agricultural industry. Meanwhile, the ability of MKH to stimulate the osteogenic activity of osteoblasts

may possess the potential to become an alternative for osteoporosis treatment. Hence, it may has the potential to become an alternative for osteoporosis treatment, making it a safe and effective medication for millions worldwide.

1.6 Graphical Abstract



CHAPTER 2

LITERATURE REVIEW

2.1 Honey

For thousands of years, honey has been utilised as a component in traditional medicine. Honey is produced and distributed primarily throughout the world by honeybees of the genus *Apis* (mainly *Apis mellifera* and to a lesser extent: *Apis dorsata, Apis cerana* and *Apis florea*). Honey is produced in tropical and subtropical areas not only by the mentioned bee species but also by numerous species of stingless bees that belong to the *Meliponinae* subfamily and the genera *Melipona* and *Trigona* (Garedew et al., 2004). Stingless bees are found in tropical and subtropical regions around the world, including Central and South America, Africa, Asia and northern Australia (Boorn et al., 2010).

2.1.1 Malaysian Kelulut Honey (MKH)

Trigona spp. or known as 'Kelulut' is a stingless bee species found in the Malaysian rainforest. Unlike *Apis spp.,* these bees produce kelulut honey. Kelulut bees store their honey in clusters of small resin pots toward the extremities of their nests, rather than hexagonal-shaped honeycombs (Hazirah et al., 2019). Stingless bee honey has a unique flavour and scent, is more fluid in texture, and crystallises slowly (Biluca et al., 2014). The distribution of stingless bee honey on the world market is limited due to supply, a shorter shelf life, and a lack of institutional quality standards as a result of the lack of information about the product (Guerrini et al., 2009).

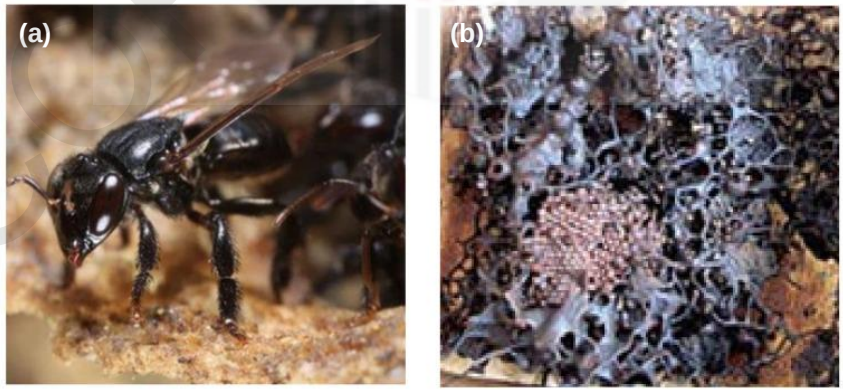


Figure 2.1: Stingless bee. (a) Stingless bee (b) hive of stingless bee
(Source: Esa et al., 2022)

2.2 Therapeutic properties of kelulut honey

The ancient people believed that kelulut honey is beneficial for the body (Badrulhisham et al., 2020). Honey that originates from *Trigona spp.* is used traditionally as a panacea against dozens of diseases and is among the most common applications in treating stomach disturbance, cough, tonsillitis, sore throat, stomach ulcers, intestinal ulcers, cold, mouth diseases, and mucus membrane, as well as in wound dressing (Badrulhisham et al., 2020; Garedew et al., 2004).

According to Zulkhairi et al. (2018) in their review, due to the outstanding chemical and biological properties of honey, scientific research has revealed that honey from *Trigona spp*. possesses antimicrobial properties (Boorn et al., 2010), antifungal properties (Chanchao, 2009), antioxidants (Budin et al., 2017; Rao et al., 2016), anticancer properties (Choudhari et al., 2013; Kustiawan et al., 2014), and anti-inflammatory properties *in vivo* and *in vitro* (Sabir & Sumidarti, 2017). Furthermore, the honey of *Trigona spp.* has better antioxidant activities than honey from *Melipona spp.* (another species of stingless bees) (Rao et al., 2016). Moreover, *kelulut* honey from *Trigona spp.* also has higher phenolic content levels compared to the honey from *Apis spp.* (Kek et al., 2014). In Malaysia, there are numerous types of honey available including *tualang, gelam, nenas, acacia,* and *kelulut* honey. *Kelulut* honey is well-known honey produced by stingless bees and it is one of the most valuable bee products.

2.2.1 Total phenolic content

Honey is high in phenolic compounds since it is made from pollen collected by bees from plants. This is most likely due to the small body size of *Trigona*, which allows her to stretch inside a larger number of flowers and produce darker colour intensity and total phenolic content compared to honey from *Apis spp.* (Kek et al., 2014).

2.2.2 Total flavonoid content

Flavonoids and other phenolic compounds from stingless honey and propolis have been associated with biological activity and traditional medicine applications (Badrulhisham et al., 2020). The total flavonoid content in stingless honey is evidently higher compared to the other types of honey such as Portuguese honey and Spanish rosemary honey, although it is considerably lower compared to the local honey such as *tualang* and *gelam* (Hazirah et al., 2019).

2.2.3 Antioxidant properties of honey

Honey is a huge source of natural antioxidants against reactive oxygen species (ROS). The quality of antioxidants in honey is difference based on geographical regions and countries (Tuksitha et al., 2018). Antioxidant properties are an element that inhibits the oxidation of other molecules. The presence of these bioactive compounds in honey able to scavenge free radicals efficiently, making it a promising source of natural antioxidants (Syed Salleh et al., 2021). As a result, the antioxidant effect of honey can surpass the bone-resorbing cytokines to prevent osteoporosis (Mohd Effendy et al., 2012).

2.2.4 Antibacterial properties of honey

Due to its antibacterial properties, honey has been used as an alternative to prevent bacterial contamination. Since honey has a low moisture content, low pH, and presence of antibacterial compounds such as peroxide (hydrogen peroxide) and non-peroxide (phenolics and bee defensin-1), it provides a protective barrier for treating and preventing microbial infection (Aspar et al., 2020; Mardhiah et al., 2022). The pH of honey is usually within the range of pH 3.2 to pH 4.5, which is deemed low enough to inhibit the growth of bacterial pathogens. Moreover, each honey also has different pH and minimum pH values for the growth of some common pathogenic bacteria such as *E.coli* (pH 4.3), *Salmonella spp*. (pH 4.0), *P.aeruginosa* (pH 4.4), and *S.pyogenes* (pH 4.5); thus, in undiluted honey, the acidity becomes a significant antibacterial factor. In fact, honey is very effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and an array of *Pseudomonas* bacteria, which are often related to wound and burn illnesses (Almasaudi, 2021; Mandal & Mandal, 2011).

Table 2.1: Physiochemical properties of MKH, tualang honey and manuka honey

Physicochemical Properties	MKH	tualang honey	manuka honey
Colour appearance	Amber brown	Dark brown	Light brown
pH	2.76 – 4.66	3.14 - 4	3.20 – 4.21
Moisture content (%)	21.52 – 33.7%	17.53% - 26.51%	18.7%
Apparent reducing sugar (%)	54.90 – 87.00	67.50 – 67.60	75.8 – 76
Fructose (g/100g)	15 – 40.20	41.732 – 44.56	40.00
Glucose (g/100g)	8.10 – 31.00	30.07 – 47.134	36.20
Maltose (g/100g)	5.73 – 27.41	4.491	1.20
Protein content (g/kg)	3.9 – 8.5	3.6 – 6.6	5.02 – 5.06
Total phenolic content (mg/kg)	477.30 – 614.7	215.7 – 1103.94	429.61
Total flavonoid content	36.3	49.04 - 185	97.62

(Source: Al-kafaween et al., 2023)

Table 2.2: Phenolic compounds of MKH, tualang honey and manuka honey

Phenolic compounds	Honey		
	MKH	tualang honey	manuka honey
Flavonoids			
Apigenin	√		
Catechine	√		
Chrysin	√		√
Galangin			√
Hesperetin		√	
Isorhamnetin			√
Pinocembrin			√
Quercetin			√
Kaempferol	√	√	√
Leptosin			√
Luteolin			√
Myricetin		√	
Naringenin		√	
Phenolic acid			
Benzoic acid		√	
Caffeic acid	√		√
Chlorogenic acid		√	
Gallic acid	√	√	√
p-Coumaric acid	√		√
Quercetin-3-O-rutinoside	√		
Syringic acid	√		√
trans-Cinnamic			√
trans-Ferulic acid			√
2-Hydroxycinnamic acid	√		
4-Hydrobenzoic acid	√		√

(Source: Afrin et al., 2020)

Table 2.3: Some of phenolic compounds MKH, tualang honey and manuka honey

Compound	Molecular Formulae	Potential Health Benefits
Apigenin	$C_{15}H_{10}O_5$	• Anti-inflammatory • Antimutagenic • Treating cardiovascular disease
Caffeic acid	$C_9H_8O_4$	• Cardiovascular disease treatment • Anti-inflammatory effects • Anticancer • Antidiabetic
Catechine	$C_{15}H_{14}0_6$	• Cardiovascular disease treatment • Antidiabetic potential • Anti-inflammatory
Chrysin	$C_{15}H_{10}O_4$	• Improves cognitive deficits and brain damage effect • Anticancer
Gallic acid	$C_7H_6O_5$	• Antioxidant • Anti-inflammatory • Cardioprotective activity • Antimutagenic • Anticancer
Kaempferol	$C_{15}H_{10}O_6$	• Cardiovascular disease treatment
p-Coumaric acid	$C_9H_8O_3$	• Anticancer activity • Improves cognitive deficits and brain damage effect
Quercetin-3-O-rutinoside	$C_{27}H_{30}O_{16}$	• Antiallergic • Anti-inflammatory • Antiproliferative • Antitumor • Cardiovascular disease treatment

(Source: Al-kafaween et al., 2023)

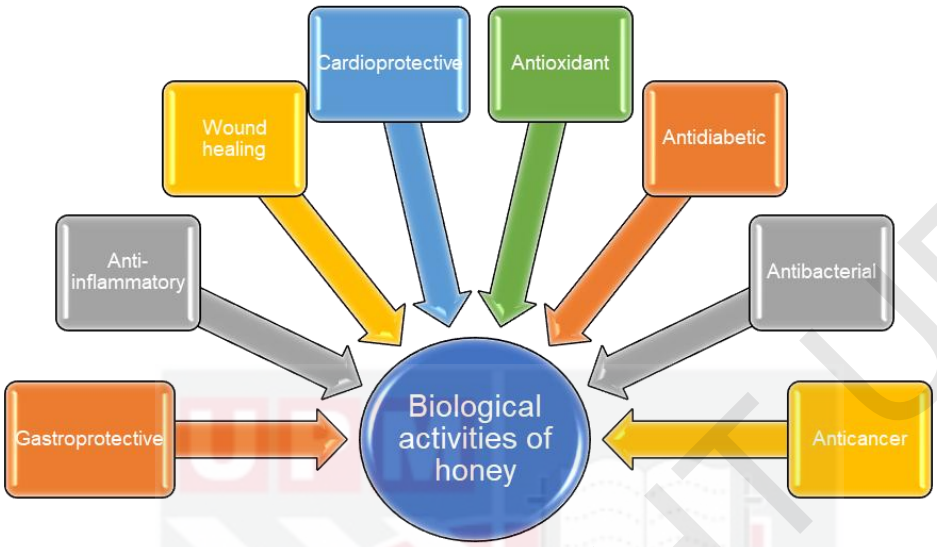


Figure 2.2: Various types of biological activities of honey
(Source: Pasupuleti et al., 2017)

2.3 Osteoporosis

Osteoporosis is the most common bone disease in humans, which has become a major public health concern around the world and a growing economic burden in our ageing society. Low bone density, degeneration of bone tissue, and disruption of bone microarchitecture are all symptoms of this condition, which can lead to weakened bones and an increased risk of fracture (Sozen et al., 2017).

Bone mineral density (BMD) is used to diagnose osteoporosis. In general, the aged person's BMD is compared to the average BMD of a 30-year-old individual of the same gender and the result is stated in standard deviation units or the so-called "T-score." If the BMD T-score is -2.5 or lower, then osteoporosis is diagnosed (Lems et al., 2011; Tu et al., 2018).

Table 2.4: World Health Organisation (WHO) criteria for the clinical diagnosis of osteoporosis

BMD T-score	Diagnosis
T-score ≥ -1	Normal
-1 > T-score > -2.5	Low bone mass
T-score ≤ -2.5	Osteoporosis
T-score ≤ -2.5 with an existing fracture	Severe osteoporosis

(Source: Lems et al., 2011; Tu et al., 2018)

Osteoporosis is affected around more than 8.9 million fractures annually, with the highest number of osteoporotic fractures occurring in Europe (Pouresmaeili et al., 2018). By 2050, it is expected that more than half of all osteoporotic hip fractures will occur in Asia (Christodoulou & Cooper, 2002). However, osteoporosis is poorly diagnosed and treated in Asia, particularly in rural areas where people choose conservative care at home over hospital surgery (Lee & Khir, 2007; Peng et al., 2006).

Hip, vertebral and wrist fractures are the most common fractures in people with osteoporosis. The cost of osteoporosis is expected to be more than £1.7 billion in nations such as England and Wales, with hip fractures accounting for 90% of the expense. In addition, osteoporosis is also expected to cost $14 billion a year in the United States (Cheung et al., 2016; Rapuri et al., 2001).

2.3.1 Osteoporosis in Malaysia

An updated hip fracture projection in Asia and the incidence of hip fractures in Malaysia will grow 3.5-fold by 2050 compared to 2018 (Ong et al., 2020). Studies had found that the prevalence of osteopenia/osteoporosis was higher among Chinese (62%) than in other ethnic groups and that 2.7% of pre-menopausal Chinese women had osteoporosis (Lee & Khir, 2007; Subramaniam et al., 2019). Besides, the overall prevalence of osteoporosis among Malaysian Chinese aged 40 years was 15.3%, with a sex-specific incidence of 11.5% for males and 18.9% for women. Age and low monthly income were also revealed to be good predictors of osteoporosis among Malaysian Chinese, while body weight was found to be a negative predictor (Chan et al., 2020; Subramaniam et al., 2019).

2.4 Aetiology of Osteoporosis

According to Tu et al. (2018), osteoporosis is categorised into two types: primary and secondary. Gender and age are usually associated with primary osteoporosis. Osteoporosis develops as the trabeculae in the bone continue to deteriorate. While a decrease in oestrogen production causes severe bone loss

in women, testosterones are also likely to reduce the activity of sex hormone-binding globulin in men and, hence, reduce BMD over time.

On the other hand, secondary osteoporosis is caused by a variety of factors, including sex and personal lifestyle. Osteoporosis in men is likely associated with alcohol intoxication, glucocorticoid therapy, and hypogonadism (Sutton et al., 2011). Women are at risk of osteoporosis due to hypercalciuria, calcium malabsorption, hyperparathyroidism, vitamin D deficiency, hyperthyroidism, Cushing's disease, and hypercalciuric hypercalcaemia. Furthermore, hyperparathyroidism and calcium metabolism problems are linked. Calcium metabolism problems and hyperparathyroidism were also found to be responsible for approximately 78% of secondary osteoporosis cases (Tu et al., 2018).

2.5 Phytochemicals treatment for osteoporosis

As osteoporosis is projected to be increased by the year 2050, the study on the phytochemical in treating osteoporosis gained interest. hence, various types of herbs had been studied to observe their potential effect as anti-osteoporotic therapy (Ong et al., 2020; Thu et al., 2017b).

2.5.1 Tongkat Ali

Eurycoma longifolia or popularly known as Tongkat Ali is a well-known herbal folk medicine in Southeast Asia, which includes the Simaroubaceae family. Its roots have been used in Asia for a long time to treat a wide range of symptoms and diseases. *E.longifolia* has gained popularity as a supplementary and alternative medicine in Western countries (Mohammad et al., 2018; Rehman et al., 2016). Other than that, the root of *E.longifolia* is also said to have medical benefits such as the ability to improve male sexuality and fertility, in addition to serving as an anti-ageing agent. Furthermore, the water-soluble extract derived from *Eurycoma longifolia* is enriched with various bioactive constituents such as phenolic components, tannins, high molecular weight carbohydrates, glycoproteins, and mucopolysaccharides. These components are believed to have the biologically active compound responsible for the observed effects in animal studies. It is suggested that these water-soluble elements act as the physiologically active substances responsible for the exhibited characteristics in the aforementioned research on animals (Shuid et al., 2011).

2.5.2 Kacip Fatimah

There are also well-known herbal medicines in Asia that are frequently used by women. The herbal plant *Labisia pumila*, also known as *Kacip Fatimah*, belongs to the Myrsinaceae family. The water extract of *L.pumila* has long been utilised

by Malay women to treat menstrual abnormalities and dysmenorrhoea. It is also used to alleviate postpartum uterine contractions and improve sexual function. Specifically, the water extract is used to cure diseases such as gonorrhoea, rheumatism, diarrhoea, and bone abnormalities. In postmenopausal women, plant extract from *L.pumila* has been demonstrated to contain estrogenic characteristics, which act as oestrogen receptor modulators. As evidenced in a study, antibody binding was shifted and anti-oestradiol antibodies were raised by the water extracts, making it similar to other oestrogens such as estrone and oestradiol (Abdullah et al., 2013).

Post-menopausal women tend to have osteoporosis due to the decreasing circulation of the oestrogen hormone. Oestrogen enhances osteoclast apoptosis while reducing osteoblast apoptosis, which will help decrease bone degradation while promoting bone growth (Jolly et al., 2018). Interestingly, the research shown that *L.pumila* has the ability to induce the release of oestrogen; thus, *L.pumila* as an alternative to estrogen replacement therapy (ERT) for managing osteoporosis in post-menopausal women. Moreover, *L.pumila* has antioxidant abilities due to the presence of active components such as ascorbic acid, anthocyanin, beta-carotene, flavonoids, and phenolic compounds. These compounds may further support bone health and overall well-being (Jolly et al., 2018; Zakaria et al., 2021) .

2.5.3 *Herba Epimedii*

In China, a medicinal plant from the *Epimedium* family is used to cure bones. *Herba Epimedii* is one of the many species in this genus. *H.epimedii* is used by the elderly in China to tonify the kidneys and strengthen the bones. In Chinese herbal medicine, this plant is always used to treat bone disorders (Zhai et al., 2013). The active components of the aqueous extract of *H.epimedii* are unknown; however, they are used to reduce bone loss. According to researchers, the total flavonoids in *H.epimedii* have pharmacological and biological effects on bone health and cardiovascular function, besides managing hormone levels, modifying immunological function, and suppressing tumour development. Because *H.epimedii* is so complex, it has been used in a variety of studies in both cell culture and animal studies, including anti-osteoporosis, oestrogen-like activity, and antitumor properties.

H.epimedii therapy had significantly influenced the differentiation of rat bone marrow-derived mesenchymal stem cells (BMSCs), with higher levels of *Runt-related transcription factor-2* (Runx2) and bone sialoprotein (BSP) mRNA and lower levels of peroxisome proliferator-activated receptor gamma-2 (PPAR-2) mRNA. *H.epimedii* treatment had also boosted alkaline phosphatase (ALP) activity and Runx2 mRNA expression in human BMSCs, while decreasing adipocyte number and PPAR-2 mRNA expression (Songlin et al., 2009).

2.6 Treatment of osteoporosis

The primary objectives of osteoporosis treatment encompass the alleviation of symptoms and the mitigation of the likelihood of subsequent fractures. There are two primary approaches to treating osteoporosis: pharmacological therapy and non-pharmacological therapy. Non-pharmacological treatments involve the use of supplements and food, whereas pharmacological treatments involve the administration of medication to either inhibit bone resorption or stimulate bone formation. Nevertheless, various challenges commonly arise during medical treatments, encompassing the undesirable consequences associated with the medication, inadequate patient response to specific drugs, the occurrence of rebound osteoporosis, and the potential acceleration of bone loss following cessation of medical interventions (Eastell, 2017; Tu et al., 2018).

2.6.1 Non-pharmacological treatments

Osteoporosis is frequently associated with inadequate calcium supplementation. As per the guidelines provided by the Institute of Medicine (IOM), it is recommended that men between the ages of 50 and 70 limit their daily intake of dietary calcium to 1,000 mg. Similarly, women aged 51 years and men aged 71 years should aim for a daily intake of 1,200 mg of dietary calcium (Sozen et al., 2017). The existing body of scholarly literature has addressed the topic of calcium and its association with the contentious issue of kidney stone development. Consequently, it is imperative to distinguish between the impacts of dietary calcium and calcium obtained through vitamin supplements. The consumption of additional calcium may potentially elevate the likelihood of developing kidney stones, whereas the intake of calcium through one's diet may serve as a protective factor against the formation of kidney stones. Therefore, it is advisable to initiate calcium supplementation subsequent to augmenting the consumption of dietary calcium (Tu et al., 2018).

Apart from calcium, the administration of vitamin D is also advocated for the management of osteoporosis. Undoubtedly, the process of calcium absorption and the maintenance of bone health are positively influenced by the presence of vitamin D. According to the Institute of Medicine (IOM), individuals between the ages of 51 and 70 are advised to consume a daily intake of 600 International Units (IU) of vitamin D. In contrast, individuals over the age of 70 are recommended to increase their daily intake to 800 IU. It is apparent that the administration of vitamin D supplements can effectively reduce the likelihood of experiencing fractures. Furthermore, recent research has indicated that there is a positive correlation between higher monthly doses of vitamin D and an increased risk of falling. Consequently, it is advisable to prescribe lower doses of vitamin D on a regular basis (Tu et al., 2018).

While it is advised to incorporate both calcium and vitamin D into one's diet for the purpose of promoting skeletal health, there is a lack of evidence supporting

the notion that vitamin D in isolation can effectively contribute to the treatment and prevention of osteoporosis (Pavone et al., 2017). The absorption of calcium from the intestine occurs through a mechanism that is dependent on vitamin D and involves both active transport and passive diffusion. The active transport mechanism plays a crucial role in maintaining calcium homeostasis due to its inverse relationship with dietary intake. In addition, it should be noted that vitamin D plays a crucial role in maintaining calcium balance and regulating parathyroid hormone levels. This is achieved through its ability to enhance the absorption of dietary calcium in the intestines and improve the functioning of proximal muscles, especially in cases where there is a deficiency of this vitamin. Furthermore, it is worth noting that vitamin D has the ability to enhance osteoblastic activity. The acceleration of osteoporosis progression in the elderly is often associated with a prevalent deficiency. Specifically, an oestrogen deficiency commonly observed in the postmenopausal state primarily impacts trabecular bone. This effect is attributed to alterations in osteoclastic activity, as noted by (Geddes & Inderjeeth, 2013).

In addition to the implementation of dietary supplements, modifying one's lifestyle can also contribute to a reduction in the likelihood of experiencing fractures. According to Dawane & Dhande (2016), the risk of fractures can be mitigated through the reduction of alcohol and caffeine intake, as well as by cessation of smoking among individuals who smoke. The consumption of alcohol in excessive amounts is widely believed to be associated with an increased risk of osteoporotic fractures and reduced bone density. Consequently, it is probable that reducing alcohol intake would be an effective approach in addressing osteoporosis (Berg et al., 2008).

In contemporary times, the consumption of coffee has emerged as a popular and fashionable practise among individuals in the workforce, particularly those who fall within the age bracket of 25 years and below. Nevertheless, it is worth noting that an excessive intake of caffeine has been associated with an elevated rate of bone loss and an increased susceptibility to osteoporosis and osteoporotic fractures (Kim, 2014; Rapuri et al., 2001). Moreover, a multitude of studies have demonstrated a correlation between coffee consumption and a diverse range of health issues. Nevertheless, based on epidemiological research, the consumption of coffee has been associated with a reduced risk of various chronic ailments, such as type 2 diabetes, Parkinson's disease, and liver cirrhosis (Kim, 2014).

The relationship between smoking and the onset of osteoporosis has been extensively studied, with Shahab (2012) highlighting smoking as a significant contributing factor to its development. This association has been well-established in the literature. Numerous studies have established a correlation between tobacco consumption and a decrease in bone density. The potential long-term effects of nicotine replacement therapy and other pharmacological smoking cessation therapies on bone dynamics have not been extensively investigated. However, gaining a deeper comprehension of the influence of nicotine and non-nicotine components of cigarettes on bone health would enable

the development of more effective smoking cessation strategies for individuals with a bone disease who desire to quit smoking (Yoon et al., 2012).

Furthermore, engaging in physical activities, such as walking, can effectively mitigate and manage osteoporosis by diminishing the likelihood of fractures. Nevertheless, there has been a lack of thorough investigation into the impact of exercise therapy on fractures. As a result, exercise interventions for individuals with osteoporosis have predominantly demonstrated a decrease in factors associated with the risk of fractures, such as a decrease in the probability of experiencing falls and/or an increase in BMD. Besides, mobility impairments such as impaired coordination and muscle strength have also been evidenced in clinical trial outcomes because they are the risk factors for falls and fractures (Body et al., 2010).

2.6.2 Pharmacological treatments

Pharmacological therapy is used to reduce the risk of fractures. Antiresorptive (bisphosphonates, oestrogen agonists and antagonists [EAAs], oestrogens, calcitonin, and denosumab) or anabolic (teriparatide) medications are used to treat osteoporosis. Anabolic medications enhance bone creation rather than resorption, while antiresorptive medications mainly slow bone resorption. Despite the fact that certain therapies have comparable indications, it is important to understand that the Food and Drug Administration (FDA) does not authorise all osteoporosis drugs to treat postmenopausal osteoporosis, osteoporosis in males, or glucocorticoid-induced osteoporosis (Tu et al., 2018).

The most common drug used to treat osteoporosis is bisphosphonates — an analogue of inorganic pyrophosphate. They reduce bone resorption by triggering the death of osteoclasts, therefore avoiding age-related bone loss and degeneration of bone microarchitecture. Bisphosphonates containing nitrogen (such as alendronate, risedronate, ibandronate, and zoledronic acid) have the strongest antiresorptive characteristics — the most widely prescribed medications for osteoporosis treatment (Pazianas & Abrahamsen, 2016; Tu et al., 2018). The ingredients are believed to improve BMD in postmenopausal women and men with osteoporosis (Yates, 2013; Zhang et al., 2012).

Denosumab is recommended as a first-line therapy by the American Association of Clinical Endocrinologists or the American College of Endocrinology (AACE/ACE) for patients who are at high risk of fracture and are unable to take oral medicines. Denosumab is the first fully human monoclonal antibody to bind directly to the human receptor activator of nuclear factor-κB-ligand (RANKL), preventing bone resorption by suppressing the production and activation of osteoclasts. This inhibition has the potential to reverse bone degeneration and loss. Denosumab has also been approved by the FDA for treating osteoporosis in postmenopausal women at a high risk of fracture, as well as increasing bone mass in men at a high risk of fracture (Pavone et al., 2017; Tu et al., 2018).

Teriparatide (TPTD) is another medication that refers to the molecule that makes up the first 34 amino acids of the intact parathyroid hormone (PTH, 84 amino acids). Parathyroid hormone stimulates bone resorption and trabecular bone loss when administered continuously, as seen in endogenous primary hyperparathyroidism. The TPTD is anabolic and increases trabecular bone when given intermittently, such as through regular injections. Based on microcomputed tomography studies of iliac crest bone biopsies, it improves not only trabecular connectivity but also cortical thickness (Eastell & Walsh, 2017). The TPTD is also safe and convenient and serves as the only therapeutic agent that promotes the development of new bone tissues, in addition to helping correct architectural flaws in the osteoporotic skeleton and being used for 24 months in clinical trials (Lindsay et al., 2016). Although there is no evidence mentioning that it prevents hip fractures, this drug is capable of raising BMD and reducing vertebral fractures by around 70% and nonvertebral fractures by approximately 45% in postmenopausal women (Eastell & Walsh, 2017).

2.7 Bone cycle

The two main processes throughout the bone cycle process include bone modelling and remodelling. Both processes generally involve osteoblastic bone formation and osteoclastic bone resorption.

2.7.1 Bone modelling

Bone modelling, which occurs early in the development of the skeleton, modifies the size and shape of the bones. At this stage, two processes are involved: bone resorption and formation (when a bone is removed from one anatomical region) and bone formation (when a new bone is formed). The bone modelling stage is crucial for skeletal form retention throughout linear development, and it also regulates the radial growth of long bones' diaphysis. On the endosteal surface, osteoclastic bone resorption occurs at this stage. Meanwhile, osteoblastic bone production occurs on the periosteal surface, resulting in an increase in the overall diameter with age. Bone modelling is normally completed when the skeleton has matured; however, bone modelling can still happen at any age (Kenkre & Bassett, 2018; Ralston, 2017; Siddiqui & Partridge, 2016).

2.7.2 Bone remodelling

Bone remodelling is the process of replacing an old and damaged skeleton with a new one. By offering rapid access to calcium and phosphate, this procedure tries to provide a mechanism for maintaining mineral homeostasis. This cycle occurs in the Basic Multicellular Unit (BMU), which is made up of osteoclasts, osteoblasts, and capillary blood. Since BMU has a longer lifespan than the osteoblasts and osteoclasts within, it requires constant replenishment of these cells, which is handled by the osteocyte (Kenkre & Bassett, 2018; Ralston, 2017;

Siddiqui & Partridge, 2016). The five overlapping processes in the remodelling cycle are activation, resorption, reversal, creation and termination, which took 120-200 days in cortical and trabecular, respectively**.**

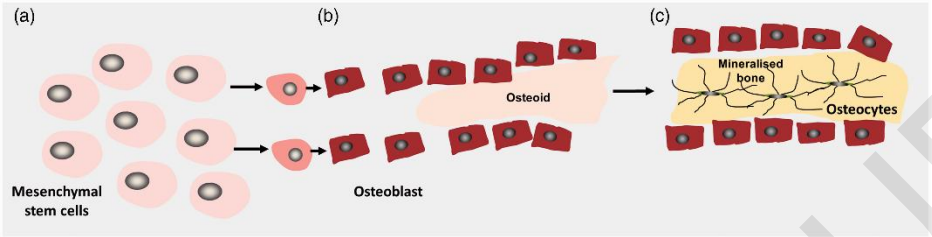


Figure 2.3: Schematic illustration BMSCs cells differentiate into osteoblasts and form bone directly. (a) BMSCs cells within the connective tissue gather and undergo differentiation into osteoblasts. (b) The mature osteoblasts produce an osteoid. (c) The osteoid undergoes mineralisation and osteoblasts ultimately mature into osteocytes and become enclosed within the newly formed bone matrix.
(Source: Kenkre & Bassett, 2018)

The first step is activation; it is crucial to ensure that bone remodelling occurs when necessary. The initial signal in targeted remodelling that refers to the removal of a specific area of damaged or old bone comes from osteocytes, which use their extensive network of dendritic processes to transmit a signal to another cells (Kenkre & Bassett, 2018). Once Runx2 activated, osteogenesis is initiated leading to the secretion of specialised gene markers like fibronectin, collagen, transforming growth factor-beta (TGFβ) receptor 1, and OPN which involved in processes like differentiation and mineralisation (Rutkovskiy et al., 2016). In this phase, osteoblast progenitors show the ALP activity and are considered preosteoblasts. In order to differentiate into mature, functional osteoblasts and reproduce, the cells first become active preosteoblasts. Next, the specialised osteoblasts start the secretion, growth, and mineralisation of the ECM. Terminal ECM formation also occurs through calcium phosphate deposition of hydroxyapatite, where osteoblasts can then mature further into osteocytes or bone lining cells (BLCs) (Crous & Abrahamse, 2021; Florencio-Silva et al., 2015).

Resorption is the second procedure, which takes around two weeks. Osteocytes are small, branched cells that create a canal network within bones. They are derived from hematopoietic stem cells (HSCs) and monocyte-macrophage lineage cells and regulate osteoblast differentiation and function. Osteocytes control osteoclast differentiation and activation, resulting in adhesion to the bone surface, the creation of a sealing zone, and the ruffled border. Initially, osteoclasts pump protons created by carbonic anhydrase II into the resorbing compartment to dissolve the bone material. The H+-ATPase then pumps H+ into lacunae and maintains electroneutrality by attaching to Cl- transport via the chloride channel. The proteolytic enzyme expressed by cathepsin K is responsible for destroying the bone matrix in the end. When mineralised, they form a complex system of osteoblasts and bone lining cells (BLCs). BLCs

remove demineralised matrix and act as a reservoir for osteoblasts in response to anabolic stimuli and tissue regeneration. To prevent excessive resorption, osteoclasts halt the resorption phase with programmed cell death (Crous & Abrahamse, 2021; Florencio-Silva et al., 2015; Kenkre & Bassett, 2018; Ralston, 2017).

The third step is a reversal, which involves switching from bone resorption to formation and takes four to five weeks. This process involves two primary events: the freshly resorbed bone surface is prepared for the deposition of a new bone matrix and the newly resorbed bone surface is prepared for the deposition of a new bone matrix. To improve osteoblast adhesion, cells of the osteoblastic lineage prepare the bone surface by removing the unmineralised collagen matrix and non-collagenous matrix. The second event is signalling, which occurs during the transition from resorption to creation to ensure that net bone loss does not occur. This process is not completely understood; however, it appears that the reversal phase cell is involved in transmitting and receiving this signal (Kenkre & Bassett, 2018; Ralston, 2017).

The formation process of bone takes four months and involves osteoblast production, secretion of a type I collagen-rich matrix, and osteoid mineralisation regulation. Hydroxyapatite crystals are deposited among collagen fibrils, and systemic calcium and phosphate regulation, as well as local inhibitors like pyrophosphate and osteopontin, control the process. The relative activity of tissue-nonspecific alkaline phosphatase and ectonucleotide pyrophosphatase are the most important determinants of the inorganic pyrophosphate to phosphate ratio, which is a crucial regulator of mineralisation. The last phase is termination. After mineralisation, osteoblasts undergo apoptosis, convert into bone-lining cells or become osteocytes, which signal the end of remodeling by secreting antagonists to osteogenesis (Kenkre & Bassett, 2018; Ralston, 2017; Siddiqui & Partridge, 2016).

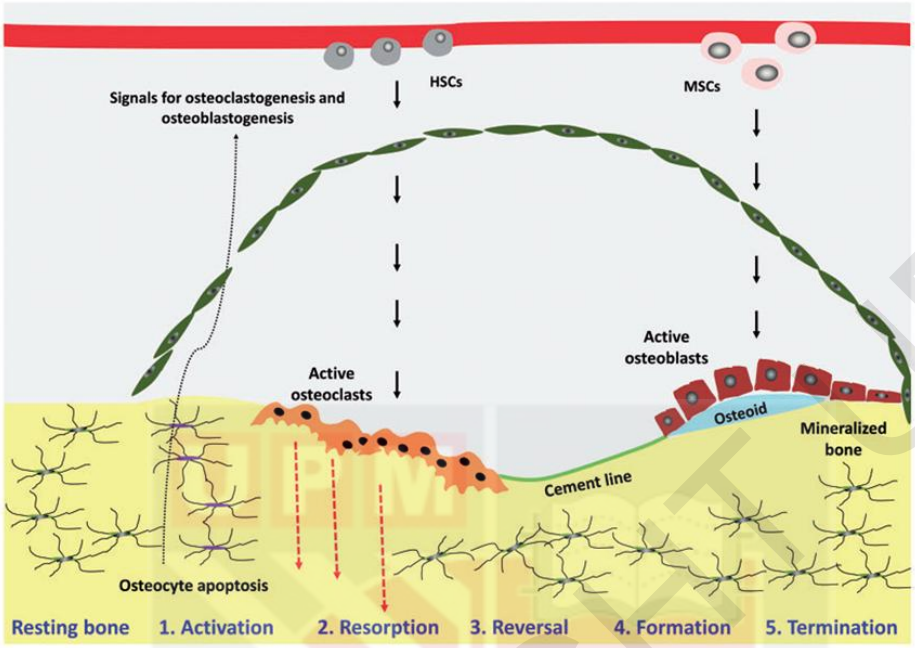


Figure 2.4: Schematic diagram of the bone remodelling cycle. The diagram illustrating the different phases of: activation, resorption, reversal, formation and termination.
HSCs - haemopoietic stem cells and MSCs - mesenchymal stem cells.
(Source: Kenkre & Bassett, 2018)

2.8 Oxidative stress effects on bone health

Oxidative stress refers to an imbalance between the synthesis and accumulation of ROS in cells and tissues, as well as the ability of a biological system to detoxify these reactive products. Many diseases, including bone disorders, have been related to oxidative stress and osteoporosis is one of the most common diseases (Domazetovic et al., 2017). In elderly osteoporosis, oxidative stress is caused by a decrease in glutathione (GSH, γ-glutamyl-cysteinyl-glycine) levels and defensive antioxidant abilities, while secondary osteoporosis is a result of intestinal chronic diseases, as well as impaired intestinal absorption of antioxidants in the diet. The activation of enzymes that produces ROS causes oxidative stress in osteoporosis, which is caused by bone inflammatory processes and long-term treatment with steroidal anti-inflammatory medicines (Domazetovic et al., 2017; Sendur et al., 2009).

Reduction in skeletal mass appears to be associated with increased osteoclastic and decreased osteoblastic activity. Osteoblasts are inhibited by oxidative stress. Oxidative stress suppresses the differentiation of a marrow stromal cell line (M2-10B4) and a preosteoblast cell line (MC3T3-E1) by measuring ALP as an early differentiation marker. Furthermore, oxidative stress decreases

mineralisation in these cell lines. Antioxidants such as trolox and pyrrolidine dithiocarbamate are used to prevent the effects of ROS (Abdollahi et al., 2005).

2.8.1 Antioxidant roles in bone health

Antioxidant supplementation is essential to counter oxidative stress and, as a result, helps prevent bone loss. Evidently, in recent studies, vitamin E or tocotrienol and *L.pumila* are the two natural compounds that can help prevent osteoporosis. Both natural compounds have antioxidant properties (Nadia et al., 2012; Nazrun et al., 2012). Antioxidant properties in honey, as well as its bone-protective properties, have seen a resurgence of interest in recent years. Compared to hormone replacement treatment, postmenopausal women who ingested *tualang* honey at 20 mg/ml for 4 months were found to have identical bone densitometry results (Mohd Effendy et al., 2012). Based on the study by Zaid et al. (2010), daily consumption of *tualang* honey for two weeks was found to increase bone density in female ovariectomised rats. Furthermore, it was also found to increase bone trabecular structure in ovariectomised rats' trabecular structure more than calcium treatment (Zaid et al., 2012).

2.9 Gene expression during osteogenesis

Bone formation is a complicated biological process involving multiple tightly controlled gene expression patterns of bone-related proteins. In both *in vivo* and *in vitro* environment, the expression of bone-related proteins during bone formation has been examined. In both *in vivo* and *in vitro* environment, the expression patterns of bone-related proteins are time-dependent. As explained by Choi et al. (2011), during bone formation, the expression of cell cycle or growth-related genes (histone, c-fos, c-myc) and extracellular matrix (ECM) genes [*collagen type 1 alpha-1* (CoLα1)] fibronectin and transforming growth factor-β1 would increase and this is followed by mineralisation-related genes such as osteocalcin (OCN), osteoprotegerin (OPG) and *bone sialoprotein* (BSP).

Osteoblasts solely secrete the OCN gene — a gamma-carboxyglutamate protein. It binds calcium ions to the bone and is the most abundant non-collagenous protein in the bone tissue. Osteocalcin (OCN) expression is promoted with Runx2, which is important in the differentiation of osteoblast. The OCN gene is a multifunctional bone-derived hormone that influences a variety of physiological and developmental processes. Based on the outcomes of the study, the deficiency of OCN in mice resulted in increased bone mass and the absence of OCN promoted bone growth without interfering with resorption. Thus, the OCN level is one of the indicators of mineral deposition (Khotib et al., 2021). However, from a different perspective, Manolagas (2020) stated that OCN did not affect bone production (or resorption) or bone mass in either the oestrogen-sufficient or oestrogen-deficient states. Rather, OCN is required for the parallel alignment of biological apatite crystallites with collagen strands.

Osteoprotegerin (OPG) is a protein found in a variety of tissues, including bone tissue. The tumour necrosis factor receptor OPG and its ligand RANKL are both important in bone resorption activity. In the degenerative phase of the bone tissue, the OPG/RANKL/RANK system plays a critical role. When RANKL and RANK interact, a signalling pathway is activated, which controls gene expression by activating nuclear factor-B (NF-B) (Khotib et al., 2021). OPG secretion protect bone osteoclast resorption through binding to RANKL; by binding to RANKL and preventing it from binding to RANK, OPG protects bone from excessive resorption. The OPG/RANKL ratio is used to determine the prevention of osteoclastogenesis. Thus, the relative concentrations of RANKL and OPG in bone is a significant marker of bone mass and strength (Tobeiha et al., 2020).

In the early phases, Runx2 is necessary for osteoblast differentiation. It controls the expression of numerous genes involved in or linked to osteoblast differentiation in general. According to *in vitro* studies, Runx2 enhances osteoblast proliferation and stimulates proliferation through the MAPK signalling pathway, which involves transcription, mRNA stability, and translation (Kawane et al., 2018; Ling et al., 2017). Target genes must interact with the regulatory site and their promoters in order for Runx2 to stimulate the production of osteogenic genes. Runx2 is essential to regulate the expression of multiple osteoblast marker genes in osteoblasts and to induce the expression of various osteoblast marker genes in non-osteoblastic cells, including CoLα1, BSP, OPN, and ALP (Bruderer et al., 2014; Ling et al., 2017).

Collagen is the most abundant protein present in the bone matrix. The component of Colα1 entails about 90% of the extracellular matrix (ECM). Collagen is typically essential for the growth and differentiation of various types of cells, including osteoblasts. Besides, the collagen protein plays an important role in the formation of structural integrity and mechanical resistance to the bone tissue. In this regard, Colα1 and osteoblasts are dependent on each other. The Colα1 gene is also activated and synthesised by osteoblasts by linking Runx2 to the promoter of CoLα1. However, in mesenchymal stem cells, Colα1 has been shown to stimulate the production of osteoblastic genes. As a result, Colα1 is frequently employed as a component of bone grafts to promote tissue regeneration in damaged bones (Khotib et al., 2021; Maehata et al., 2009).

Bone sialoprotein (BSP) is strongly expressed by osteoblasts and hypertrophic chondrocytes, which are abundant in primary bone forming sites. The BSP is a highly glycosylated non-collagenous phosphoprotein that is expressed during the start of hard connective tissue mineralization, which occurs late in the osteoblastic differentiation process (Bouet et al., 2015; La Noce et al., 2021). BSP overexpression promotes the production of osteoblast-related genes and the development of mineralised nodules in culture. On the other, suppressing osteoblast marker expression in osteoblasts using a specific shRNA result in a significant decrease in matrix mineralisation (Bouet et al., 2015). According to Lin et al. (2020), a decrease in BSP may cause a decrease in cementum deposition, long bone length, and cortical thickness, as well as a slower rate of bone formation. Reduced BSP expression in osteoblasts, on the other hand,

inhibits the expression of osteoblast markers and results in a considerable reduction in matrix mineralisation. Therefore, BSP is one of the powerful mineralisation nucleators and matrix-associated signals that promotes differentiation and improved mineralised matrix production (Bouet et al., 2015; Khotib et al., 2021; Lin et al., 2020).

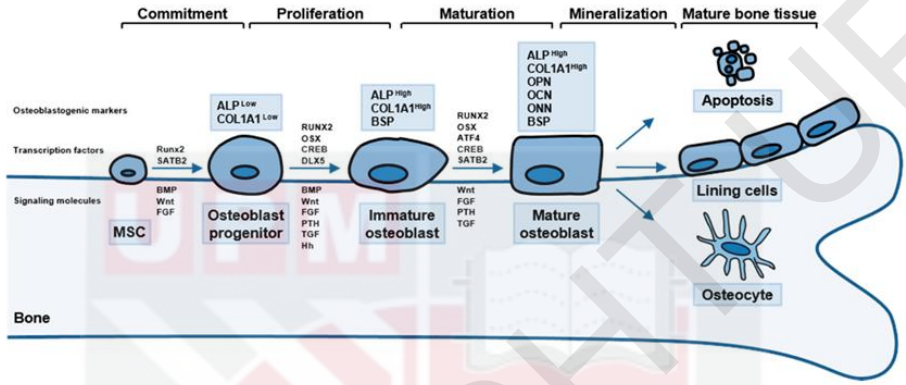


Figure 2.5: Schematic representation of gene expression in the osteoblast differentiation.
(Source: Amarasekara et al., 2021)

2.10 Osteoblast

Osteoblasts are derived from two Greek words, which combined in the form are cells with mononuclear cells that synthesise bones. Osteoblasts are cells that produce bones in response to the mechanical forces on our bones as well as additional growth stimuli such as hormones. In bone formation, osteoblasts work in groups as linked cells during the process of bone development. Mesenchymal stem cells are responsible for the generation of osteoblasts, which are highly specialised cells that have reached a terminal stage of differentiation. Osteoblasts exhibit a notable reduction in the synthesis of thick, cross-linked collagen and specific proteins such as OCN and OPN. These proteins are essential components of the organic matrix found in bones. Hence, it is crucial to emphasise the significance of the initial phase of osteoblast differentiation, as it is during this stage that the premature osteoblast undergoes a transformation into a fully developed osteoblast (Florencio-Silva et al., 2015).

2.10.1 Preosteoblast cells

The preosteoblast cell is an osteoblast precursor cell generated from the calvaria of *Mus* musculus (mouse). Different variants of these strains have been identified and used as a model system in bone biology to select for variable degrees of

osteogenic capacity. The most convenient and physiologically relevant system for studying transcriptional control in calvaria osteoblast is the MC3T3-E1 subline from osteoblast. These cell lines are ideal for researching osteoblast development *in vitro,* especially in ECM signalling.

2.11 Viability and proliferation of cells

2.11.1 MTT assay

The 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTT) assay is a colorimetric assay. The MTT assay is a cheap and fast method for measuring cytotoxicity. The MTT assay has also become a well-known method, including for the treatment of honey on the cells (Abel & Baird, 2018).

The MTT assay operates on the principle that viable cells maintain constant mitochondrial activity. This activity leads to the conversion of the tetrazolium salt MTT into formazan crystals, whose concentration can be measured in a homogeneous manner. It compares OD values of treated cells to untreated cells in drug sensitivity experiments (Riss et al., 2004). The MTT assay is suitable for evaluating drug sensitivity in both established cell lines and primary cells. The assay assesses drug sensitivity as the concentration needed to suppress cell growth by 50% in comparison to untreated controls (IC_{50}) in dividing cells. Drug sensitivity for non-dividing cells, such as primary cells, is assessed by measuring an increase in cell death over the normal cell loss that occurs in untreated settings (LC_{50}). When comparing the results to the controls, it is not issue that the control median cell survival is 100% in some cell types, such as fresh acute myeloid leukemia cells (van Meerloo et al., 2011).

2.11.2 Cell proliferation using BrdU assay

Bromodeoxyuridine (BrdU) is a thymidine analogue used to label proliferating and migrating cells in human and animal organs. The indirect enzyme-linked immunosorbent assay (ELISA) kit for BrdU works by detecting BrdU incorporation into newly synthesized DNA of actively dividing cells. BrdU is added to cell cultures in microtiter plates during the last 2 to 24 hours of cultivation, and the cells are then fixed, permeabilised, and denatured. The anti-BrdU detector monoclonal antibody is added and allowed to bind to the incorporated BrdU. After washing away the unbound antibody, a horseradish peroxidase-conjugated goat anti-mouse antibody is introduced, leading to a color change of the chromogenic substrate (TMB) in proportion to the amount of BrdU in the cells. The intensity of the color is measured at 450nm using a spectrophotometer. This sensitive and simple assay can be applied to high sample throughput and provides additional information, such as cell number,

morphology, and cell antigen analysis, all from a single culture. The BrdU assay is a valuable tool for studying cell proliferation and related characteristics (Lequeux et al., 2011).

CHAPTER 3

METHODOLOGY

3.1 Malaysian Kelulut Honey (MKH) sample and MC3T3-E1

3.1.1 Malaysian Kelulut Honey (MKH)

Malaysian Kelulut Honey (MKH) was obtained from local *kelulut* apiarists in Cameron Highlands, Pahang. The identification was performed by the apiarists based on the area and floral availability at the location of the apiary. Stock solution (100%) of honey was subsequently kept in the transparent glass bottle and stored in the laboratory at room temperature (25°C to 28°C).

Figure 3.1: Malaysian *Kelulut* Honey

3.1.2 Preparation of fresh media

Complete culture media (CCM) was prepared by mixing Minimum Essential Media Eagle (αMEM) (Nacalai Tesque, Japan), 10% of fetal bovine serum (FBS) (Tico Europe, Netherland), and 1% antibiotic and antifungal, i.e., streptomycin (Nacalai Tesque, Japan) and amphotericin B (Sigma Aldrich, USA) in the culture media.

3.1.3 Culturing of preosteoblasts

Murine preosteoblast cell line (MC3T3-E1)[1] was obtained from Riken, Japan and cultured in CCM and incubated in a CO_2 incubator (Eppendorf, UK) supplemented with 5% CO_2 at 37ºC and 95% atmosphere humidity. The cells was cultured from passage 0 after obtained from the supplier and were used at 80%-90% confluency for seeding and treatment throughout the experiment. Passages 6-10 were also used throughout the experiment.

3.1.4 Changing the media

The cells were retrieved from the incubator and the used media was removed from the flask and washed using phosphate-buffered saline (PBS) (Thermo Fisher, USA) to remove the impurities and dead cells in the flask. Next, the CCM was added to the flask. The cells were then monitored using an inverted microscope (Olympus, Japan) and incubated at 37°C/95% humidity/5% CO_2.

3.2 Seeding of MC3T3-E1

3.2.1 Cell counting

The cells were retrieved from the incubator and the used media was discarded and washed in the flask using PBS to remove the impurities and dead cells in the flask. The cells were added with 1 mL of trypsin (Nacalai Tesque, Japan) for cell detachment and incubated in the incubator for 3 minutes. Subsequently, 2 mL of CCM was added to the flask and transferred to the cells into a 15 mL centrifuge tube. The cells were then centrifuged (Hettich, US) at 1200 rpm for 10 minutes at 4°C. Next, the supernatant of the cells was discarded and the CCM was added to the cell pallet and re-suspended the cells thoroughly.

The trypan blue exclusion method was used to assess cell count and viability. A 10 µL sample of cell suspension was mixed on a parafilm with 10 µL of trypan blue solution (Sigma Aldrich, USA) (0.4% in PBS) and transferred to a Neubauer haemocytometer. Viable cells were counted and the concentration of viable cells in the suspension was calculated. The number of cells was calculated using the formula provided.

[1] Appendix 1: Riken BRC Cell Bank of MC3T3-E1 cells.

$$\frac{n}{2} \times 10^4 \times 2 \times V_1 = \frac{\text{number of cell needed}}{\text{volume of media for each well (mL)}} \times \text{total volume of media (mL)} \quad \text{Eq. (3.1)}$$

Where n = number of counted cells
V_1 = volume of the media (mL)

3.3 Preparation of samples for the treatment of cells

Prior to the treatment of the cells, MKH was prepared freshly using the final concentration of 20% v/v using differentiation media (DM). This concentration was chosen as the lowest feasible concentration achievable through serial dilution. Various concentrations of MKH were prepared from the stock solution and sterilised with a 0.2 µm syringe filter (Bioflow, Malaysia). Subsequently, MC3T3-E1 cells were treated with different MKH concentrations (0.0025% v/v, 0.005% v/v, 0.01% v/v, 0.02% v/v, 0.05% v/v, and 0.1% v/v) which not toxic to the cells. The stock of calcium solution for the positive control was prepared at a final concentration of 1.0 mg/mL by diluting lactic acid hemi-calcium salt (Sigma Aldrich, USA) in DM before being filtered for sterilisation purposes. The culture media was subsequently replaced every 3-4 days throughout the experiment.

3.4 Assessment of cell viability and cell proliferation

3.4.1 Preparation of MTT reagent

3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTT) (Sigma Aldrich, USA) reagent powder was weighed in a microcentrifuge tube, dissolved in the PBS solution, and mixed using a vortex (VELP Scientifica, Italy). The solution was filtered using a 0.2 µm syringe filter and the final MTT concentration in the assay was 0.5 mg/mL. The centrifuge tube was wrapped with aluminium foil and the solution was kept in the chiller for long-term use.

3.4.2 MTT assay protocol

The cells were cultured in DM in a 96-well plate (NEST Scientific, Japan) at $1.0X10^4$ cells/well and treated with multiple concentrations of test substances for 24, 48, and 72 hours. MTT reduction assay was used to measure the cell viability.

Tetrazolium salt of MTT will be reduced by dehydrogenase in metabolically active cells to produce coloured formazan that can be measured at 570 nm. After

24 hours of MKH treatments, 20 µL MTT reagent was added to the cells before being incubated (37°C/95% humidity/5% CO_2) for 4 hours before the solution in the well was discarded. The developed formazan was then dissolved by adding 100 µL of dimethyl sulfoxide (DMSO) (Amresco, USA). Subsequently, the absorbance of the formazan was measured by a spectrophotometer microplate reader (SpectraMax Plus 384, Molecular Device, USA) at a wavelength of 570 nm. The proliferation percentage of MC3T3-E1 cells was calculated as below:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treatment (570nm)}}{\text{Absorbance of control (570nm)}} \text{ x } 100 \qquad \text{Eq. (3.2)}$$

Results from this assay were used to determine the effective concentration of MKH used in another assay.

3.4.3 BrdU assay

To assess the effects of MKH on osteoblast proliferation, 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 cell proliferation in the samples were measured by Bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit (Millipore©, Merck, Germany). Bromodeoxyuridine (BrdU), an analogue of the DNA precursor thymidine, was incorporated into newly synthesised DNA by actively proliferated cells and detected by staining them with a specific anti-BrdU antibody that can be measured spectrophotometrically at 450 nm.

Following a period of treatment with MKH, 20 µL of diluted BrdU solution was added to the appropriate wells at least 2 hours before the end of the incubation period. The wells were carefully aspirated, and 200 µL of fixing/denaturing solution was added, incubating at room temperature for 30 minutes. Afterward, the fixing solution was aspirated, and the plate was washed three times with 1X wash buffer, with the buffer being aspirated after the final wash. Next, 100 µL/well of diluted antibody was added and incubated at room temperature for 1 hour. The plate was washed three times again before 100 µL/well of goat anti-mouse IgG, peroxidase conjugate solution, was added and incubated for 30 minutes at room temperature. The solution was aspirated, and the plate was washed three times with wash buffer. The final wash was performed by flooding the entire plate with distilled water once. Lastly, 100 µL/well of TMB peroxidase substrate was added, and the plate was incubated for 30 minutes at room temperature in the dark. The plate was then read using a spectrophotometer microplate reader at 450nm. The proliferation percentage of MC3T3-E1 cells was calculated as below:

$$\text{Cell Proliferation (\%)} = \frac{\text{Absorbance of treatment (450nm)}}{\text{Absorbance of control (450nm)}} \text{ x } 100 \qquad \text{Eq. (3.3)}$$

Results from this assay were used to determine the effective concentration of MKH used in another assay.

3.5 Preparation to induce osteoblastic differentiation

To induce osteoblastic differentiation, the cells were cultured in the differentiation media (DM) that consists of CCM plus 100 nM dexamethasone (Nacalai Tesque, Japan), 0.05 µM ascorbate-2-phosphate (Nacalai Tesque, Japan), and 10 mM β-glycerophosphate (Santa Cruz Biotechnology, USA) and incubated at 37°C/95% humidity/5% CO_2. The cells were used at 80%-90% confluency for seeding and treatment throughout the differentiation and mineralisation experiment.

3.6 Osteoblastic differentiation and mineralisation assays

The effects of MKH on the osteoblastic differentiation was qualitatively assessed by using osteoblast phenotype assays, namely colony-forming assay, alkaline phosphatase (ALP) assay, calcium deposition assay, collagen deposition assay, level of osteocalcin (OCN) and osteoprotegerin (OPG) expression, and qualitatively based on histochemical staining for deposition of colony/nodule, ALP, calcium, collagen, and inorganic phosphate.

To enhance the differentiation and mineralisation of MC3T3-E1, the cells were cultured in DM. The cells were seeded into 24-well plates (NEST Scientific, USA) at 20,000 cells/well and cultured with an optimum concentration of test substances for 14 and 21 days, respectively. At the end of the incubation period, osteogenic differentiation and mineralisation assay were assessed by the methods below.

3.6.1 Colony-Forming Assay

The colony-forming assay was carried out to assess the ability of osteoblasts to form a colony or nodule by using crystal violet staining with some modifications (Thu et al., 2017a). After 14 and 21 days, the cultured cells' monolayer was rinsed with PBS twice and fixed using 100% ethanol (HmbG, Germany) for 10 minutes. The cells were then washed with PBS thrice to reduce the colour of the background and stained with 0.2% crystal violet (Sigma Aldrich, USA) (dissolved in 10% ethanol) for 10 minutes. Subsequently, the plates were washed with PBS several times and air-dried at room temperature. The images were then

observed under a phase-contrast microscope (Olympus, Japan) with a digital camera attached.

For the quantification analysis of the nodule formation assay, the stained cells were dissolved with 10% acetic acid (Systerm, Malaysia). Following that, the staining solution was shaken gently before being transferred to a 96-well plate and measured at 590 nm of wavelength using a spectrophotometer (SpectraMax Plus 384, Molecular Device LLC, USA). The absorbance value was used to analyse the data.

3.6.2 Alkaline Phosphatase (ALP) Assay

The alkaline phosphate (ALP) activity of the cells was determined by the ALP staining assay. The monolayer of cultured cells was rinsed with PBS and fixed with 100% ethanol for 60 seconds. They were then rinsed using washing buffer (0.05% Tween 20 in PBS) and stained with freshly prepared ALP staining solution for 5-10 minutes using SIGMAFAST™ BCIP®/NBT (Sigma Aldrich, USA) by diluting 1 tablet in 10 mL of autoclaved water. Afterwards, the cells that were stained were washed with the washing buffer and left to air-dry. Subsequently, they were observed under a phase-contrast microscope equipped with a digital camera.

For ALP quantification, Alkaline Phosphatase Assay Kit (Colorimetric) (ab83369) (Abcam, USA) was used. After 14 and 21 days, the cells were harvested and supernatant from the cells was collected. The p-Nitrophenyl Phosphate (pNPP) standard for the experiment was prepared freshly according to the manual given. To assess ALP activity, a sample background control was included, and a 20 µl stop solution was used to halt ALP activity in these samples. Following this, all samples were mixed by pipetting, and each well containing the sample and background control received 50 µL of 5 mM pNPP solution, except for the standard wells. For the pNPP standard wells, 10 µL of ALP enzyme solution was added and mixed. The plate was then incubated at 25°C for 60 minutes, protected from light. The enzyme transformed the pNPP substrate into colored p-Nitrophenol (pNP) in equal amounts. To stop the reaction, a 20 µL stop solution was added to the samples, and the plate was gently shaken before measuring the absorbance at 405 nm using a spectrophotometer. Finally, the ALP activity in MC3T3-E1 cells was determined using the provided calculation in the kit.

$$\text{ALP activity } (\text{U/mL}) = \frac{\text{B}}{\Delta\text{T}\times\text{V}} \qquad \text{Eq. (3.4)}$$

Where B = amount of pNPP in sample from standard curve
ΔT = reaction time (minutes)
V = volume of sample (mL)

3.6.3 Calcium Deposition Assay

The ability of the cells to form calcium mineralised nodules was determined by Alizarin red assay. The cultured cells' monolayer was rinsed with PBS and fixed using 100% ethanol for 40 minutes. They were then stained with alizarin red S staining solution (2 g Alizarin Red S [Sigma Aldrich, USA] in 100 mL of distilled water, pH 4.1-4.3, filtered by 0.2 µm syringe filter) for 45 minutes at room temperature in the dark with gentle agitation. The stained cells were carefully removed, rinsed with distilled water, and air-dried before being observed under a phase-contrast microscope with a digital camera attached.

The quantification of calcium deposition was carried out based on the method advocated by Siddiqui and Arshad (2014). Specifically, the cells were de-stained with 100 mM cetylperidium chloride (Sigma Aldrich, USA) for 1 hour in the dark to release calcium bound alizarin red into the solution. Following that, the extracted stain was transferred to a 96-well plate and measured at 570 nm. The absorbance value was used to analyse the data.

3.6.4 Collagen Deposition Assay

The collagen formation of the cells was determined by the collagen deposition assay described by Siddiqui and Arshad (2014). The monolayer of cultured cell was rinsed with PBS twice before being fixed using 70% of ethanol for 1 hour. Subsequently, they were stained with collagen staining solution (1 mg/mL Sirius red in 1.3% saturated picric acid [Sigma Aldrich, USA]) for 1 hour at room temperature. The stained cell layers were then extensively washed with 0.01N hydrochloric acid (HCl) (Systerm, Malaysia) to remove all non-bounded dye and further rinsed with distilled water for the last wash. The cells were left to air-dry before observation under a phase-contrast microscope equipped with a digital camera. Finally, an additional extensive washing step with 0.01N HCl was carried out to ensure complete removal of any non-bound dye from the stained cell layers.

The quantitative analysis of the collagen deposition assay was performed as the stained cells were dissolved in 0.2 mL 0.1N sodium hydroxide (NaOH) (Merck, Germany) in a shaker for 30 minutes. Next, absorbance was measured calorimetrically at 550 nm using a spectrophotometer against 0.1N NaOH serving as a blank and the percentage of collagen content was calculated:

$$\text{Collagen content (\%)} = \frac{\text{OD (treatment)}_{550\text{nm}} - \text{blank}_{550\text{nm}}}{\text{OD (control)}_{550\text{nm}} - \text{blank}_{550\text{nm}}} \text{ x } 100 \qquad \text{Eq. (3.5)}$$

3.6.5 Inorganic-Phosphate Deposition Assay

The ability of the cells to form phosphate mineralised nodules was determined by *von Kossa* staining as described by Thu et al. (2017) with some modifications. The cultured cells' monolayer was rinsed with PBS before being fixed by ice-cooled 70% ethanol for 1 hour. Subsequently, the cells were rinsed with distilled water. They were also stained with 5% silver nitrate solution (Nacalai Tesque, Japan) under ultraviolet (UV) until the deposits turned dark brown. The cells were further rinsed with distilled water before the un-reacted silver was rinsed with 5% sodium thiosulfate (Nacalai Tesque, Japan) for 5 minutes at room temperature and air-dried before being observed under a phase-contrast microscope with a digital camera attached.

3.6.6 Osteocalcin (OCN) Assay

Osteocalcin (OCN) formation of the cells was assessed using an OCN ELISA kit (Mouse OC/BGP (Osteocalcin) ELISA Kit, Elabscience) (Elabscience, USA). To begin the assay, a standard working solution of 100 µL was prepared for the first two columns of the plate. Subsequently, 100 µL of cell lysate samples was added to the remaining wells. The plate was then sealed and incubated at 37°C for 90 minutes. Afterward, the liquid was removed, and 100 µL of biotinylated detection antibody working solution was added to each well. The plate was sealed again and incubated for 1 hour at 37°C. Following this, the liquid from each well was aspirated, and a wash buffer was used for three washes. Next, 100 µL of horseradish peroxidase (HRP) conjugate working solution was added to each well. The plate was covered and incubated for 30 minutes at 37°C. Another round of washing was performed. Then, 90 µL of substrate reagent was added to each well. The plate was covered with a new plate sealer and incubated for 15 minutes at 37°C in the dark. Finally, the reaction was stopped by adding 50 µL of stop solution to each well, and the optical density (OD) value of each well was measured at 450 nm.

3.6.7 Osteoprotegerin (OPG) Assay

Osteoprotegerin (OPG) formation of the cells was assessed by using an OPG ELISA kit (Mouse Osteoprotegerin [OPG] ELISA Kit, Elabscience) (Elabscience, USA). For the assay, 100 µL of the standard working solution was added to the first two columns of the plate, while the cell lysate samples (100 µL) were added to the other wells. The plate was then covered with the provided sealer and incubated for 90 minutes at 37°C. After the incubation, the liquid was removed from the wells, and 100 µL of biotinylated detection antibody working solution was added to each well. The plate was covered again and incubated for 1 hour at 37°C. Subsequently, the liquid from each well was aspirated, and a wash buffer was used for three washes. Next, 100 µL of HRP conjugate working solution was added to each well. The plate was covered and incubated for 30

minutes at 37°C. Following another round of washing, 90 µL of substrate reagent was added to each well. The plate was then covered with a new plate sealer and incubated for 15 minutes at 37°C in the dark. Finally, the reaction was stopped by adding 50 µL of stop solution to each well, and the optical density (OD) value of each well was measured at 450 nm.

3.7 Osteoblastic differentiation and mineralisation of bone-specific mRNAs expression

To assess the effects of MKH on the expression of genes of the osteoblastic differentiation, RNA analysis was carried out using RNeasy Mini Kit (Qiagen, USA) and followed by DNA synthesis using QuantiNova™ Reverse Transcription Kit (Qiagen, USA). The process was continued using QuantiNova® SYBR Green RT-PCR Kit (Qiagen, USA) containing the assays for osteogenesis-associated genes, namely *Runt-related transcription factor 2* (Runx2), *collagen type 1 alpha-1* (COLα1), *bone sialoprotein* (BSP), and housekeeping gene β-actin on Mastercycler® RealPlex2 (Eppendorf, Germany).

3.7.1 Extraction of RNA

The cells were harvested in the vessel and 350 µL of Buffer RLT was added. The lysate was centrifuged (Eppendorf, Germany) for 3 minutes at maximum speed. Then, 1 volume of 70% ethanol was added to the lysate and thoroughly mixed by pipetting. Subsequently, 700 µL of the mixture was transferred to the RNeasy Mini spin column, which was placed in a 2 mL collection tube. The sample was centrifuged for 15 seconds at 8000 g, and the flow-through was discarded. The process continued by adding 700 µL of buffer RW1 to the RNeasy Mini spin column, followed by another centrifugation at 8000 g for 15 seconds, and discarding the flow-through. Then, 500 µL of Buffer RPE was added to the RNeasy Mini spin column, and the sample was centrifuged again at 8000 g for 2 minutes to remove any remaining impurities. The flow-through was discarded, and the sample was centrifuged at full speed for 1 minute to dry the membrane. To elute the RNA, a new 1.5 mL collection tube was placed beneath the RNeasy Mini spin column, and 50 µL of RNase-free water was added directly to the spin column membrane. The sample was centrifuged for 1 minute at 8000 g, effectively eluting the RNA. The concentration of the RNA in the samples was then measured using a Nanodrop photometer (Implen, Germany).

3.7.2 Synthesis of DNA

The DNA synthesis procedure was prepared according to the QuantiNova™ Reverse Transcription Kit (Qiagen, USA). RNA template was thawed in the ice together with gDNA removal mix and reverse transcription enzyme. Likewise, reverse transcription mix and RNase-free water were thawed at room temperature. Subsequently, the genomic DNA removal reaction was prepared on ice as follows: 2 µL of gDNA removal mix, RNA template, and RNase-free water. The samples were then incubated for 2 minutes at 45°C in a water bath (Memmert, Germany) before they were immediately placed on ice. After that, 20 µL of the reverse transcription master mix was prepared on ice and mixed well as per the following: reverse transcription enzyme 1 µL, reverse transcription mix 4 µL, and template RNA 15 µL.

The samples were incubated for 3 minutes at 25°C for the annealing step and incubated for 10 minutes at 45°C for the reverse-transcription step. Finally, for the inactivation of the reaction, the samples were incubated for 5 minutes at 85°C and the samples were kept at -20°C for long-term storage.

3.7.3 Qualitative Reverse transcriptase polymerase chain reaction (qRT-PCR) analysis

The following entails the number of samples used in the RT-PCR: 10 µL of SYBR green master mix, 1µL of cDNA template, 1 µL of reverse primer (700 nm), 1 µL of forward primer (700 nm), and 7.5 µL of RNase-free water. The reaction mix was prepared at room temperature prior to the hot start-up. The sequence of the primers of Runx2, CoLα1, BSP and the housekeeping gene β-actin gene is presented in Table 3.1.

The amplification and detection processes were conducted using the Mastercycler® RealPlex2. The procedure involved an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation, annealing, and polymerisation. Denaturation occurred at 95°C for 20 seconds, followed by primer annealing at 60°C for 30 seconds, and DNA extension at 72°C for 30 seconds. Negative controls without target DNA were included for each gene, and all extractions were performed in triplicate. To analyse the PCR results, the relative quantitative analysis of each gene was expressed as a ratio of PCR products to β-actin. The $2^{-\Delta\Delta CT}$ method was employed to determine the gene expression levels.

Table 3.1: List of forward and reverse primers

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Runx2	ACTCTTCTGGAGCCGTTTATG	GTGAATCTGATGTTTGTG
COLa1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
BSP	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT
β-actin	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

(Source: Liu et al., 2016; Liu et al., 2019)

3.8 Statistical analysis

The results were presented as means ± SEM, and statistical significance was assessed using one-way analysis of variance (ANOVA) with post-hoc Dunnett tests for multiple comparisons. Additionally, student t-tests were used for time-dependent analyses between groups. A p-value of less than 0.05 was considered statistically significant. The analysis was carried out using SPSS (IBM SPSS Statistics version 20) and GraphPad Prism 9, with all procedures performed in triplicate (n = 3).

3.9 Study design

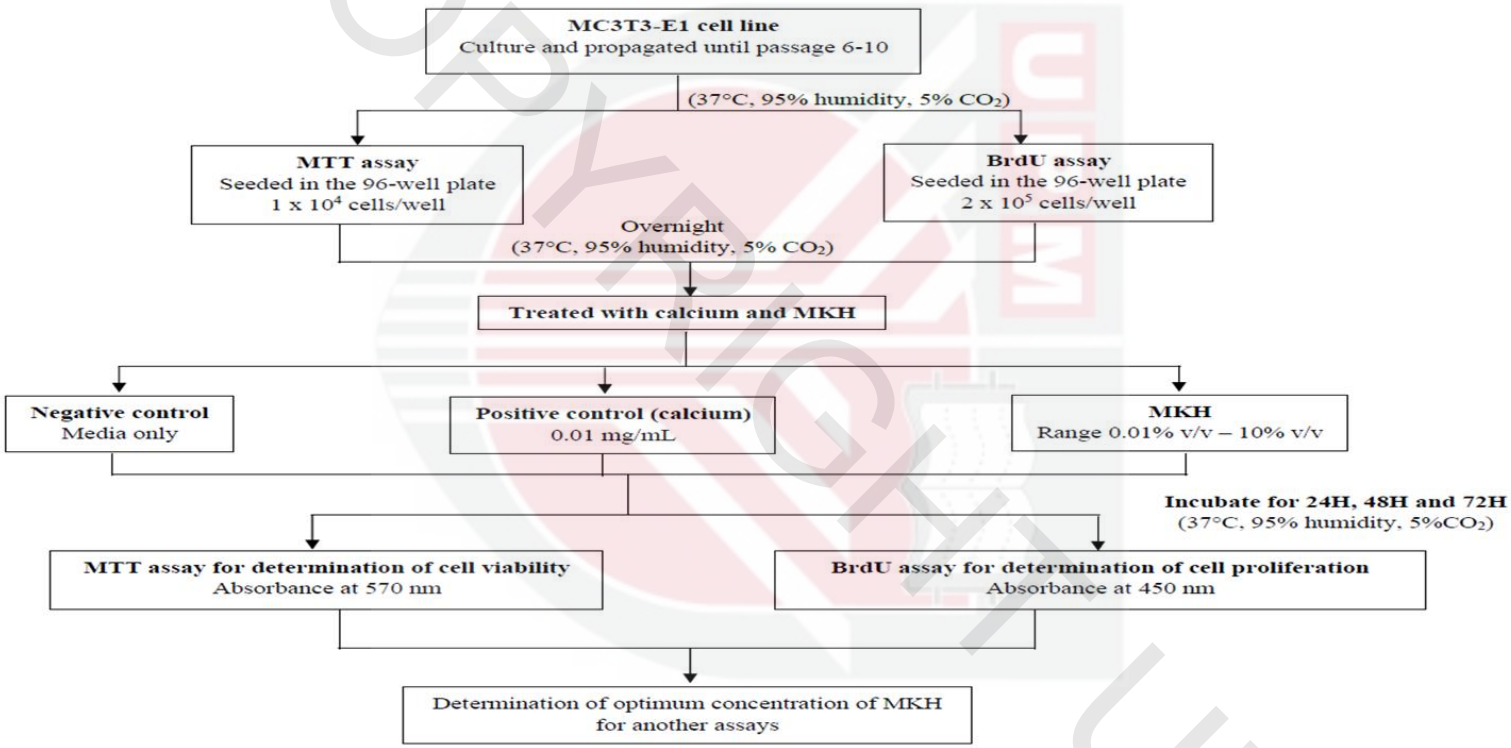


Figure 3.2: Flowchart of Optimisation of MKH concentration

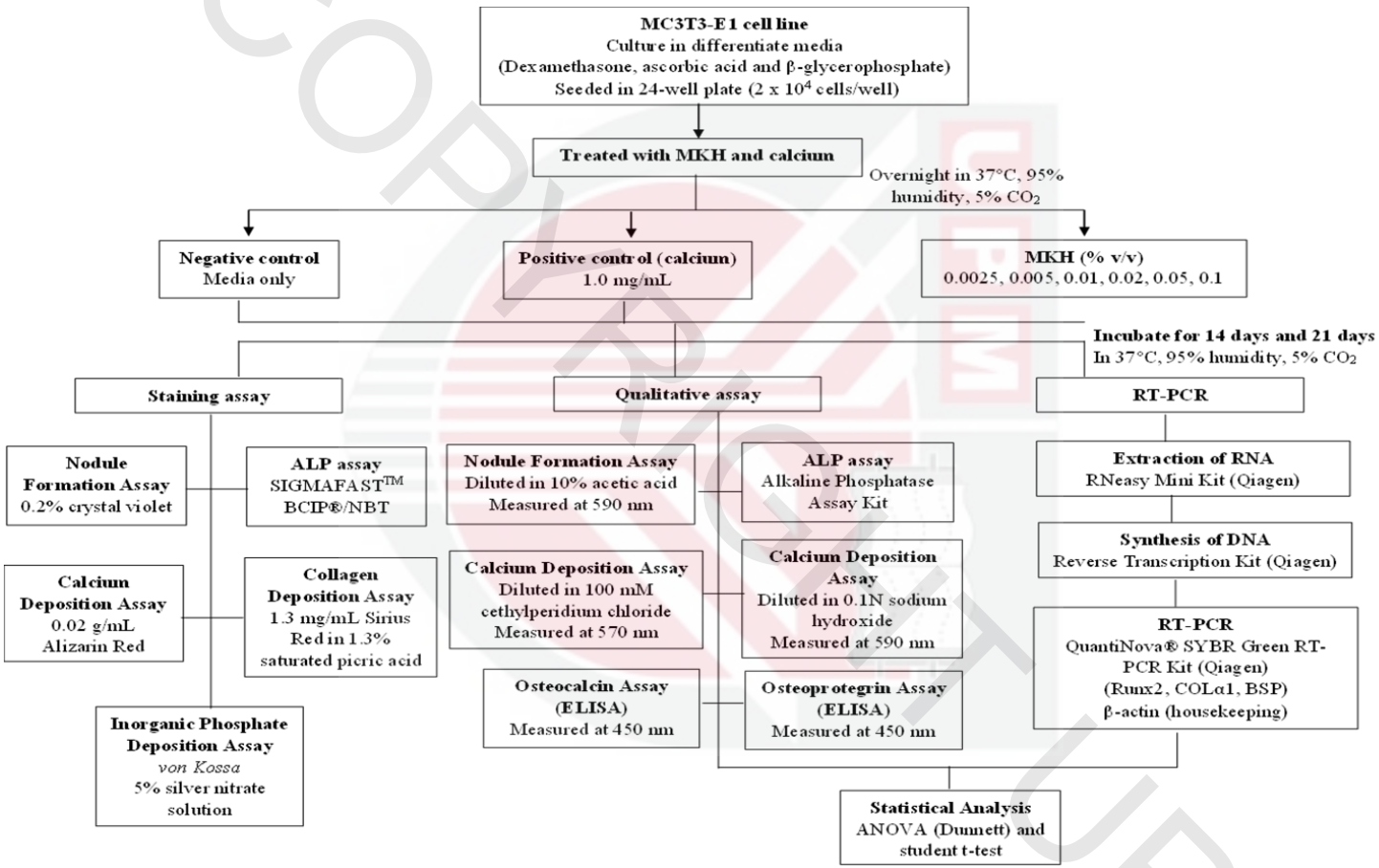


Figure 3.3: Flowchart of Differentiation and Mineralisation Assays of MC3T3-E1 Cells

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Assessment of the cytotoxicity, viability and proliferation of MKH on MC3T3-E1 cells

Cell viability and proliferation are important in osteoporosis because, in a healthy skeleton, the bone tissue constantly undergoes remodelling to prevent an imbalance in synthesising the new bone by osteoblasts and the degradation of the bone matrix by osteoclasts (Macías et al., 2020). Thus, the risk of osteoporosis can be reduced if the proliferation of osteoblasts increases. Furthermore, the formation of osteoblasts is important in bone modelling and remodelling since the former osteoblasts were trapped in the bone matrix. In particular, remodelling depends on fine-tuned crosstalk between these protagonists. This ensures that the amount of bones resorbed by osteoclasts equals the number of bones formed by osteoblasts, thereby ensuring the maintenance of bone mass (Föger-Samwald et al., 2020).

To investigate the cytotoxicity and viability effects of Malaysia *Kelulut* Honey (MKH), 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTT) assay was used on the treated MC3T3-E1 cells. The cells treated with various concentrations of MKH from 0.01% v/v to 10% v/v, including the untreated group as the negative control. The MTT assay is a widely recognized and the simplest method for assessing cell viability, making it highly advantageous compared to other techniques. This colourimetric assay offers sensitivity, quantification, and reproducibility, allowing it to detect cell viability, proliferation, and activation effectively. The assay relies on tetrazolium salts to measure the rate of mitochondrial metabolism, indirectly reflecting the number of viable cells present. Specifically, in metabolically active cells, the tetrazolium salt is reduced by mitochondrial dehydrogenases, particularly succinate dehydrogenase, resulting in the formation of water-insoluble purple formazan crystals. These formazan crystals can be easily measured using spectrophotometers once solubilised. The total amount of formazan produced is directly proportional to the number of viable cells in the culture (Rai et al., 2018; van Meerloo et al., 2011; Vega-Avila & Pugsley, 2011).

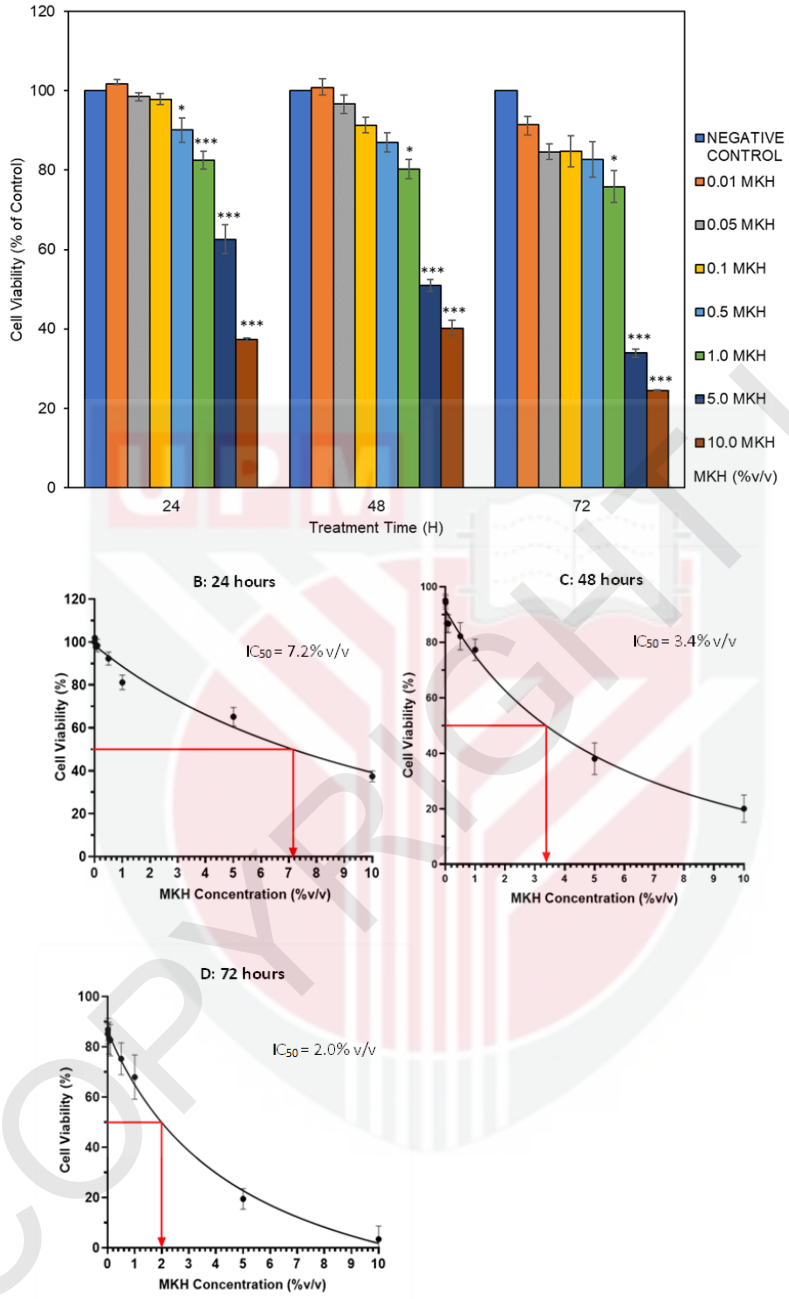


Figure 4.1: Viability of MC3T3-E1 cells under the influence of various MKH concentrations when treated at different times. B, C and D showed IC_{50} values at different times of treatment were extrapolated from respective curves at cell viability (y-axis) of 50%. The IC_{50} of MKH was shown for 24 hours, 48 hours and 72 hours of treatment at 7.2%, 3.4% and 2.0% v/v of MKH concentration, respectively. Data were expressed (n=3) as the mean percentage of viability ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control for 24 hours, 48 hours and 72 hours.

The percentage of viable cells was assessed at different time points, namely at 24 hours, 48 hours and 72 hours. Based on the Figure 4.1 (A), after 24, 48 and 72 hours of treatment, there was no difference shown in increasing viability of MC3T3-E1 cells between the MKH and the negative control observed. The viability of MC3T3-E1 cells decreased as the MKH concentrations increased. The 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 of 0.01% v/v to 0.1% v/v were not cytotoxic to MC3T3-E1 cells.

Furthermore, according to Figure 4.1 (B, C, and D), the half-maximal inhibitory concentrations (IC_{50}) of MKH were reported at a concentration of 7.2% v/v after 24 hours, 3.4% v/v after 48 hours and 2.0% v/v after 72 hours of the experiment, respectively. Hence, the concentrations of MKH that is less than and equal to 0.1% MKH were selected to be used for other procedures in this study.

The result of viability obtained in this study was similar to the other studies of honey conducted on different types of cells. Other than that, the result showed that the reduction of viability occurred in different types of cell lines such as keratinocytes (Nordin et al., 2020), lymphoblastoid (Hazirah et al., 2019; Ooi et al., 2021), and RAW 264.7 murine macrophage cell lines (Ooi et al., 2021) in a dose-dependent manner when treated with increasing concentration of MKH.

Beside that, the experiment continued by investigating the effect of MKH on the proliferation of MC3T3-E1 cells by using a bromodeoxyuridine (BrdU) assay that was carried out at time intervals of 24 hours, 48 hours and 72 hours. The concentrations of MKH used in this experiment were as the same as in the previous cytotoxicity and viability studies. The proliferation activity of MC3T3-E1 cells was assessed using a bromodeoxyuridine (BrdU) assay, which entailed a synthetic nucleoside analogue of the DNA precursor thymidine (5-Bromo-2'-deoxyuridine). This assay's principle mechanism of action is to take advantage of the process during cell division. Before cell division occurs during cell proliferation, DNA in the cells must be duplicated. As such, the BrdU assay was appropriate for determining the number of DNA replications since it measured the amount of pyrimidine analogue of BrdU incorporated into the DNA rather than the original molecule of thymidine. The establishment of techniques to quantify DNA synthesis *in vivo* and cultured cells by immuno-cytochemistry (counted labelled) or by BrdU enzyme-linked immunosorbent assay (ELISA) has been facilitated by the development of monoclonal antibodies directed against BrdU. In both techniques, either *in vivo* or *in vitro,* the amount of BrdU incorporated into cultured cells in microplates was quantified colourimetrically in a microplate reader. This is the fastest quantifying DNA synthesis method in many samples (Vater et al., 2020; Vega-Avila & Pugsley, 2011). The number of DNA replications showed proliferation activity, indicating that as DNA replication increases, cell proliferation also increases.

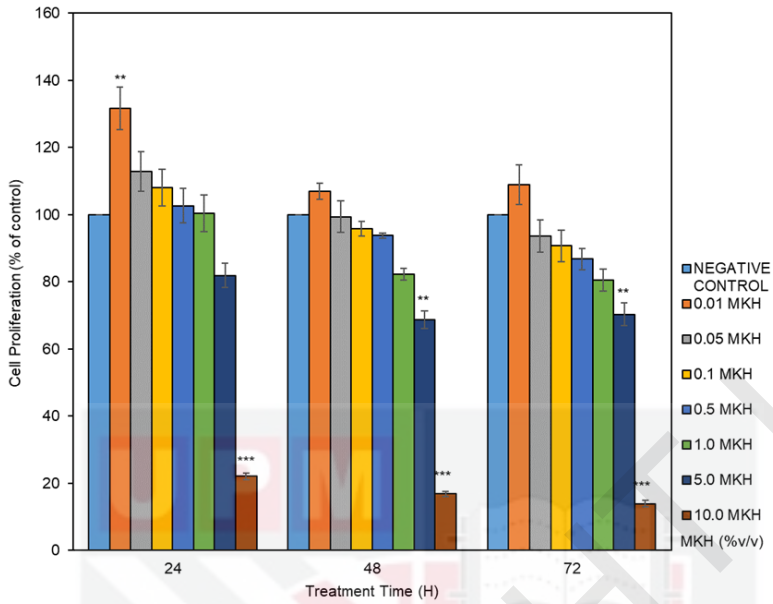


Figure 4.2: The proliferation percentage of MC3T3-E1 cells under the influence of various MKH concentrations when treated at different times. Data were expressed (n=3) as the mean percentage of proliferation ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control for 24 hours, 48 hours and 72 hours.

According to Figure 4.2, after 24 hours of treatment, the proliferation of MC3T3-E1 cells treated with MKH concentrations at 0.01% v/v until 0.5% v/v increased compared to the negative control group. However, only the proliferation of MC3T3-E1 cells treated with MKH at concentration of 0.01% v/v increased significantly ($p<0.001$) against the negative control up to 131.65%. However, there were no significant difference against negative control showed on the lower MKH concentration after 48 and 72 hours of treatment. Apart from that, the cell proliferation started to decrease when treated with 1.0% v/v until 10% v/v MKH as the MKH concentration increased. The highest MKH concentration (10% v/v) there were significant difference ($p<0.001$) against negative control observed decreasing cell proliferation in MC3T3-E1 cells after 24, 48 and 72 hours of treatment.

The study observed a parallel relationship between the percentage of proliferation and cell viability in MC3T3-E1 cells. The half maximal inhibitory concentration (IC_{50}) of MKH was reported at a concentration of 2.0% v/v after 72 hours. In addition to this, the other study proved that the use of honey at low concentrations increase the proliferation of cells. The results of this study were consistent and aligned with the findings of Yu et al. (2015), who observed the highest cell proliferation on human periodontal ligament fibroblasts (HPDLFs) when treated with 0.02% v/v honey for 72 hours. Similarly, Yusof et al. (2016) identified the optimal concentration of *gelam* honey was at 0.0015% to obtain

the highest proliferation of corneal keratocytes after 48 hours of incubation. In addition, the findings reported study by Kannan et al. (2009) also found that the highest cell proliferation on human osteoblast cell lines (CRL 1543) was observed at a low concentration (0.0195% v/v) of *tualang* honey after 72 hours of treatment. Hence, it proved that the MKH proliferate the osteoblasts at low concentrations. It was assumed that the nutritional effect of honey might enhance the proliferation caused by the carbohydrate content of honey, which is the substrate for glycolysis (Yu et al., 2015). Thus, it can be inferred that MKH has the potential to maintain the viability and proliferation of MC3T3-E1 cells.

Therefore, the lower MKH concentrations than its IC_{50} were chosen to evaluate the effects of MKH on osteoblasts viability and proliferation upon-long term exposure. Treatment with MKH with concentrations less than its IC_{50} sustained the viability of osteoblasts compared to the negative control. As expected, IC_{50} of MKH and above negatively impacted the viability of osteoblasts at all time points. This study can be supported by Nordin et al. (2020), who observed the viability of keratinocytes increased at a low concentration of kelulut honey (0.0015% v/v), besides the ability to maintain cell viability within 120 hours at that concentration. In a different study conducted by Nordin et al. (2018), who used a freeze-dried sample of MKH, they observed the viability of fibroblast cells isolated from consent patients increased after being treated with 0.024 µg/mL of MKH for 24 hours.

At higher concentrations (>1% v/v), MKH was found to be slightly toxic to MC3T3-E1 cells. Furthermore, the acidic nature of honey or hyperosmolarity could be why honey is toxic at high concentrations since the pH of honey generally is between 3.27-3.30, influencing the viability and proliferation of osteoblasts in culture (Agasi et al., 2021; Nordin et al., 2020; Yu et al., 2015). Like natural plants, a high concentration of honey could also be toxic to the cells (Kannan et al., 2009; Yusof et al., 2016). Same as the viability study, at a high MKH concentration above 1.0% v/v, MKH was observed to inhibit osteoblast proliferation. It was assumed that the acidic characteristics of MKH with high moisture content may reduce osteoblast development.

An optimal pH range is between 7.2 and 7.4 for almost all cells in the culture. Therefore, an alteration of pH in the cell culture media can impede the viability and proliferation of the cells. However, adding honey to the medium for cell culture did not alter the pH of the medium (Nordin et al., 2020). As a result, the low pH acidic nature of MKH contributes to its excellent antimicrobial properties. But unfortunately, it contributes to the inhibition of the proliferation of osteoblasts (Yap & Chin, 2019). Thus, lower MKH concentrations within the range of 0.0025% v/v to 0.1% v/v were selected throughout this experiment as these concentrations are deemed safe for assessing the differentiation and mineralisation of MC3T3-E1 cells.

4.2 Effects of MKH on the differentiation and mineralisation of MC3T3-E1 cells

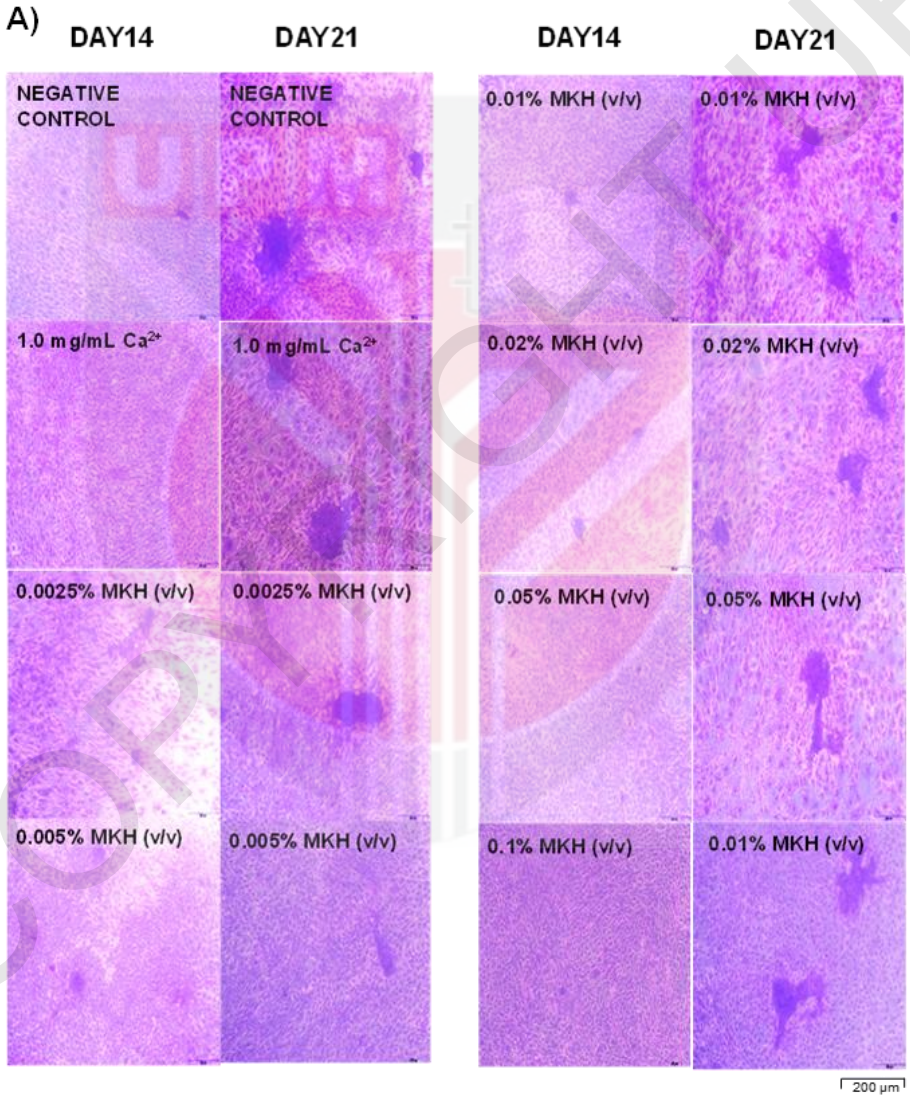
4.2.1 Effects of MKH on osteoblastogenesis activity in MC3T3-E1 cells

To stimulate the differentiation and mineralisation processes in osteogenic conditions, dexamethasone, ascorbic acid, and β-glycerophosphate were added to the culture media. Dexamethasone induces *Runt-related transcription factor 2* (Runx2), expression by FHL2/β-catenin mediated transcriptional activation and enhanced Runx2 activity by the upregulation of TAZ and MAPK phosphatase (MKP-1) (Langenbach & Handsche, 2013). Besides that, ascorbic acid plays a vital function as a cofactor in the post-translational modification of collagen molecules, which was thought to be the cause of the time-dependent increase in collagen formation (Thu et al., 2016). Furthermore, the phosphate from β-glycerophosphate serves as a source for the phosphate in hydroxyapatite, in addition to influencing intracellular signalling molecules (Langenbach & Handsche, 2013).

In order to investigate MKH's potential to promote the differentiation of pre-osteoblastic cells into osteoblasts, the MC3T3-E1 cells were treated with MKH for 14 and 21 days, following the protocol utilized in numerous osteoblastic differentiation studies. This time frame aligns with the period of maturation for osteoblasts, during which they produce collagenous and non-collagenous proteins that form the matrix of newly developed bone and facilitate the mineralization process (osteoid). This maturation process typically spans approximately 21 days (Blair et al., 2017; Liu et al., 2021). Hence, to assess the phenotypic differentiation of MC3T3-E1 cells, various parameters were studied at two time points, namely day 14 and day 21. The parameters under this study included colony formation, ALP (alkaline phosphatase) activity, and the deposition of calcium, collagen, and inorganic phosphate. These parameters aimed to evaluate the osteoblastic differentiation process and its potential enhancement by MKH treatment.

The first phenotypic differentiation studied involved colony formation, which was assessed using the crystal violet assay. This staining method allowed for the study of MKH's effects on cellular growth during the early stage of MC3T3-E1 cell differentiation. The staining assay was recognised by the appearance of dark purple spots on the cells, as in Figure 4.3 (A). The results showed that the intensity and size of dark purple spots appeared in all treatment groups, including the calcium-treated group (positive control) and negative control after exposure to the samples for 14 days and 21 days. The intensity and size of dark purple spots continued to increase until day 21, as in Figure 4.3 (A). However, the colony formation was more obvious in positive control and the cells treated with MKH at concentration of 0.01% v/v. The staining result was interpreted for the quantification analysis by diluting the staining with acetic acid to obtain the absorbance value, as shown in Figure 4.3 (B). As the intensity and size of the

dark purple spots increased, the absorbance value also increased. Thus, the colony formation in MC3T3-E1 cells also increased during mineralisation. Furthermore, Figure 4.3 (B) showed that in comparison between day 14 and day 21 of treatment within the same group, negative control and 0.01% v/v MKH showed significant differences ($p<0.01$) versus their counterparts as well as groups treated with MKH at the concentrations of 0.0025% v/v, 0.02% v/v, 0.05% v/v and 0.1% v/v showed significant difference ($p<0.05$) against their respective counterparts.



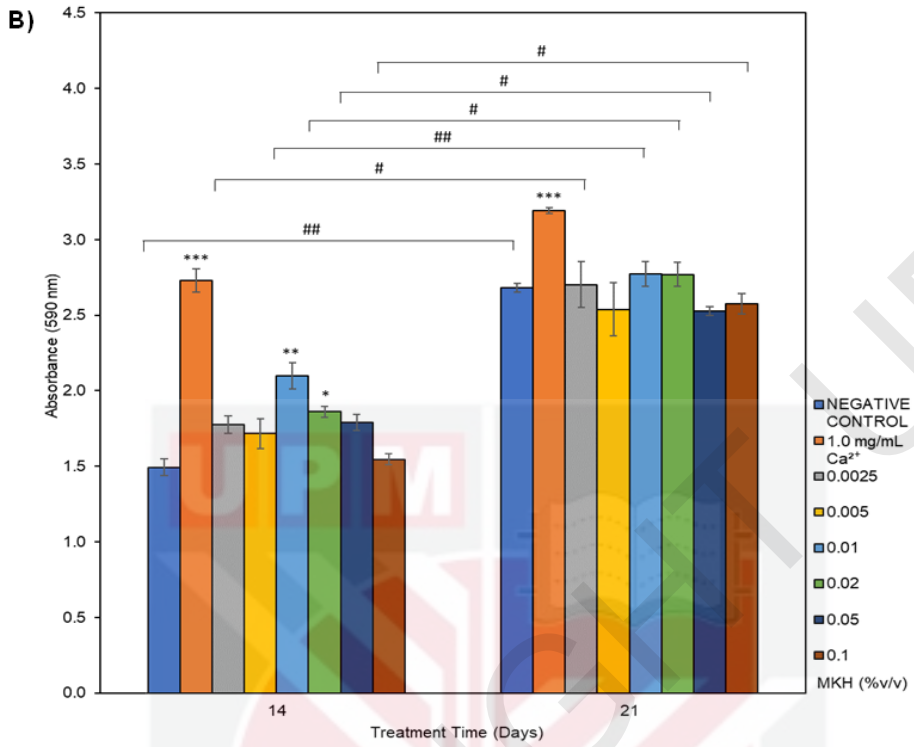
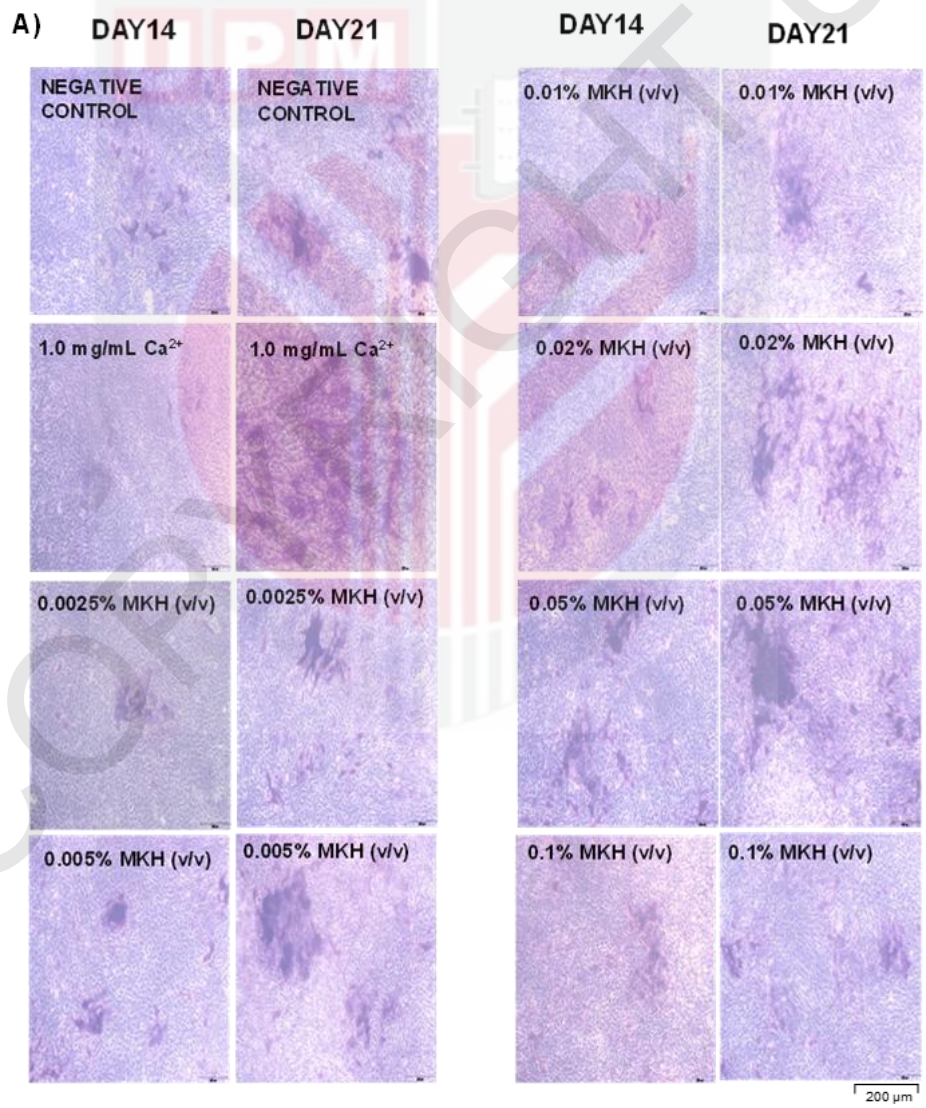


Figure 4.3: Calcification of colony formation in MC3T3-E1 cells after 14 days and 21 days. (A) representative images in size and intensity of the dark purple spots were observed in various concentrations of MKH and 1.0 mg/mL calcium-treated groups of MC3T3-E1. (B) Absorbance at 590 nm in MC3T3-E1 cells for quantification by acetic acid for 14 days and 21 days. Data were expressed (n=3) as the mean absorbance ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control at day 14 and 21. Multiple group comparisons between various concentrations of MKH were made at two-time points. #p<0.05 and ##p<0.01 were considered significant.

On day 14 of treatment, the intensity and size of the dark purple spots were significantly (p<0.001) high in the positive control, followed by MC3T3-E1 cells treated with 0.001% v/v MKH (p<0.05) when both were compared with the negative control. In the rest of the MKH-treated groups, no significant difference (p>0.05) was observed despite having a higher absorbance value compared to the negative control. Furthermore, on day 21, there was no significant difference showed by MKH-treated groups and only cells treated with calcium (positive control) had significant difference (p<0.001) showed significant difference compared to the negative control. The highest intensity and size of the dark purple spots were observed in cells treated with calcium (positive control), followed by cells treated with 0.01% v/v and 0.02% v/v MKH then followed by negative control group.

Subsequently, the alkaline phosphatase (ALP) assay was conducted, as ALP serves as one of the earliest markers for identifying osteoblastic differentiation in MC3T3-E1 cells. The ALP staining assay was recognized by the appearance of dark blue spots within the cells, and quantification was carried out using the ALP kit. As shown in Figure 4.4 (A), the size and intensity of dark blue spots in the cells observed on day 14 increased as the treatment time increased until day 21 in all treated groups, including both control groups. The size and intensity of the dark blue spots were translated to the ALP activity for quantitative analysis, as shown in Figure 4.4 (B). As the size and intensity of the dark blue spots increased, the ALP activity also increased. On day 21 of treatment, MC3T3-E1 cells treated with MKH showed a significant difference at $p<0.001$ when compared to their respective groups on day 14.



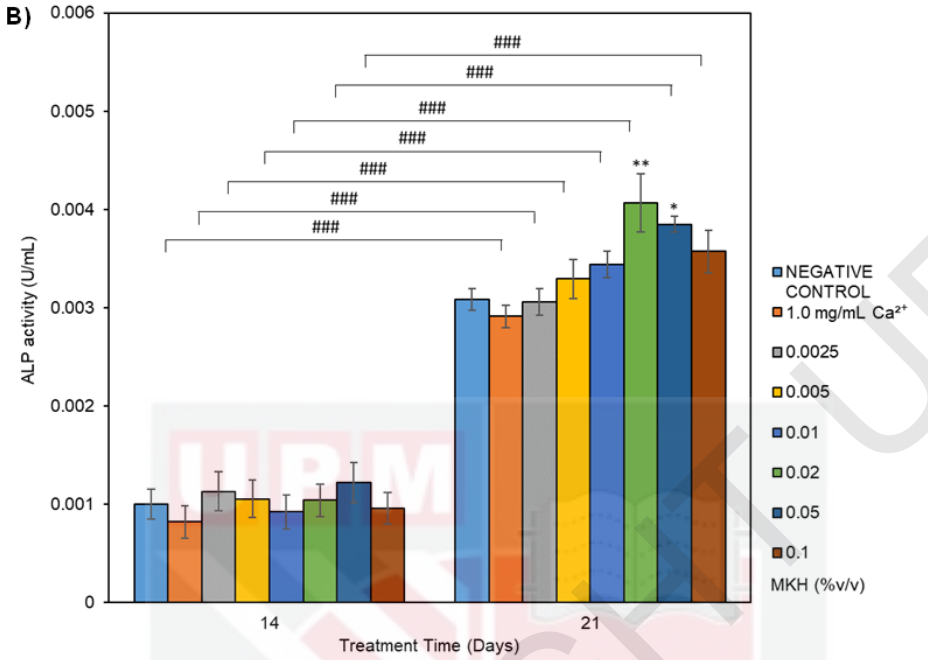


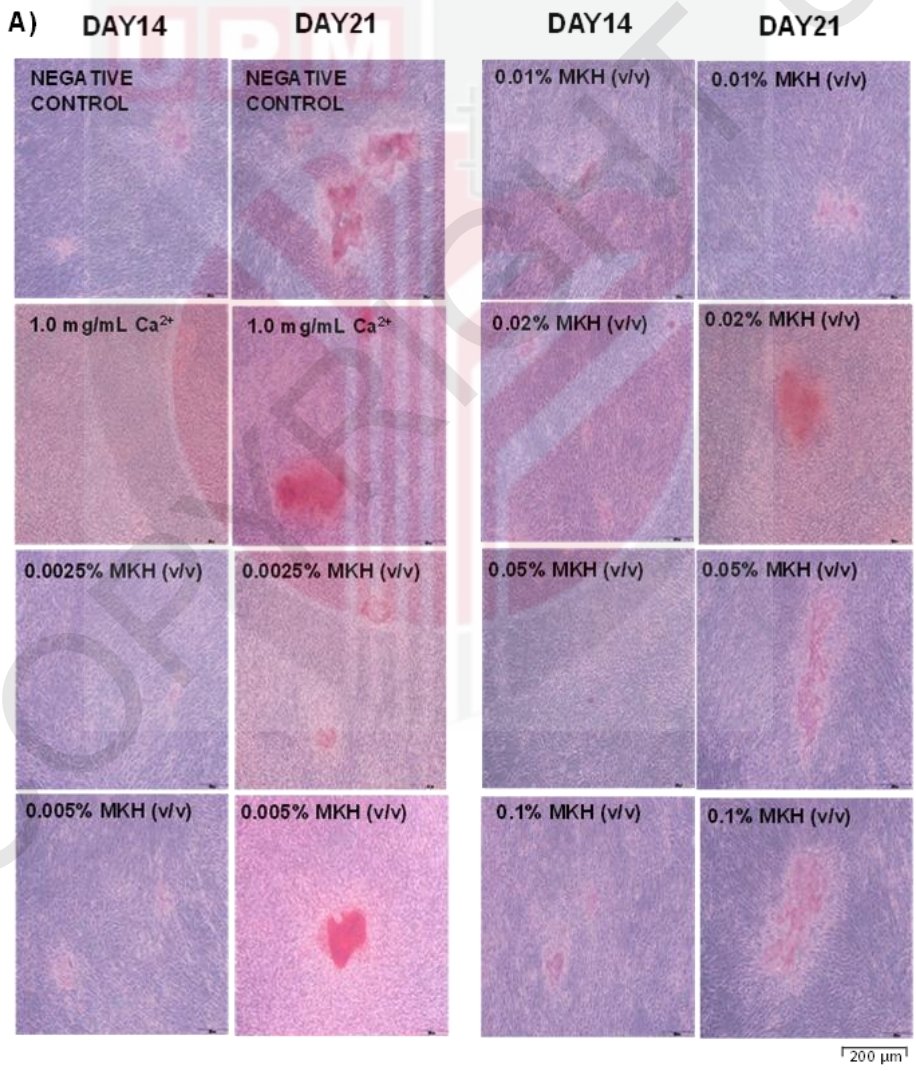
Figure 4.4: Calcification of ALP activity in MC3T3-E1 cells after 14 days and 21 days. (A) representative images in size and intensity of the dark blue spots were observed in various concentrations of MKH and 1.0 mg/mL calcium-treated groups of MC3T3-E1. (B) ALP activity in MC3T3-E1 cells for quantification by ALP kit for 14 days and 21 days. Data were expressed (n=3) as the mean absorbance ± SEM. *p<0.05 and ***p<0.001 were considered significant vs. their respective negative control at day 14 and 21. Multiple group comparisons between various concentrations of MKH were made at two-time points. $^{\#}$p<0.05 and $^{\#\#}$p<0.01 were considered significant.

On day 14 of treatment, there were no significant differences (p>0.05) shown between all treatment groups, including positive control (calcium-treated) when compared to the negative control. The highest ALP activity on day 14 was observed in cells treated with 0.02% v/v MKH followed by 0.0025% v/v MKH, 0.005% v/v MKH, 0.02% v/v MKH, negative control, 0.1% v/v MKH, 0.01% v/v MKH and positive control. However, on day 21 of the treatment, 0.02% v/v MKH and 0.05% v/v MKH showed the highest ALP activity when compared to the negative control. The ALP activity in 0.1% v/v MKH, 0.05% v/v MKH, 0.005% v/v treated cells higher than the positive control, 0.0025% v/v MKH and negative control on day 21.

Furthermore, to assess the effectiveness of MKH in promoting mineralisation (calcium deposition) in MC3T3-E1 cells, a qualitative analysis was conducted using Alizarin Red. This dye forms a deep red colour upon complexation with calcium ions in mineralised nodules. For quantitative analysis, the red colour could be dissolved by 10% cetylpyridinium chloride (CPC), resulting in a red-

coloured solution. The absorbance of this solution was directly proportional to the amount of calcium deposited in the cells.

The photomicrograph results illustrated in Figure 4.5 (A) showed that the size and intensity of the deep red spots on day 21 were larger than on day 14 in all treatment groups, including positive and negative controls. Furthermore, the size and intensity of the deep red spots gradually increased until day 21. However, according to Figure 4.5 (B) in comparison between day 14 and day 21 of treatment within the same group, there was no significant difference ($p>0.05$) shown in the size and intensity of the deep red spots when compared to their counterparts on day 21 except for the calcium-treated group ($p<0.05$).



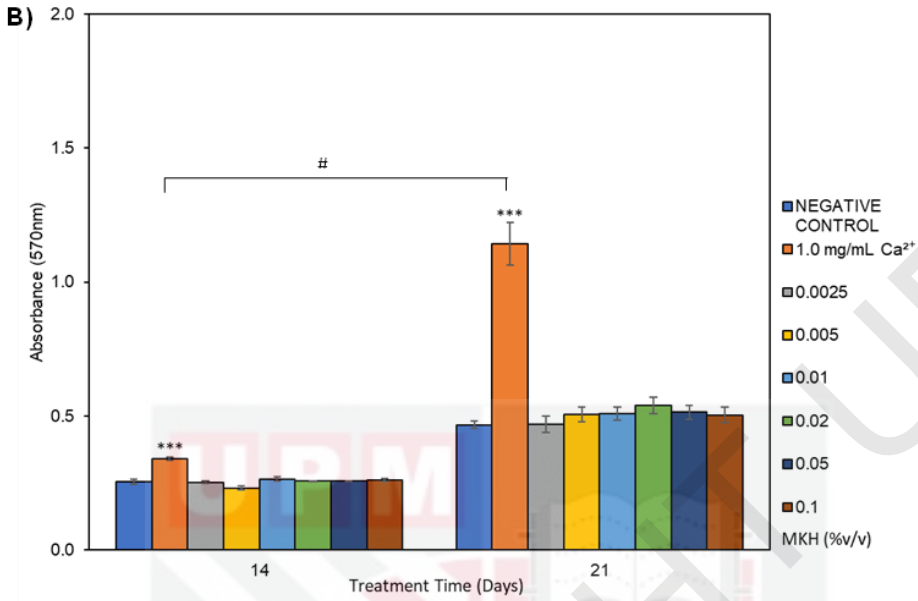
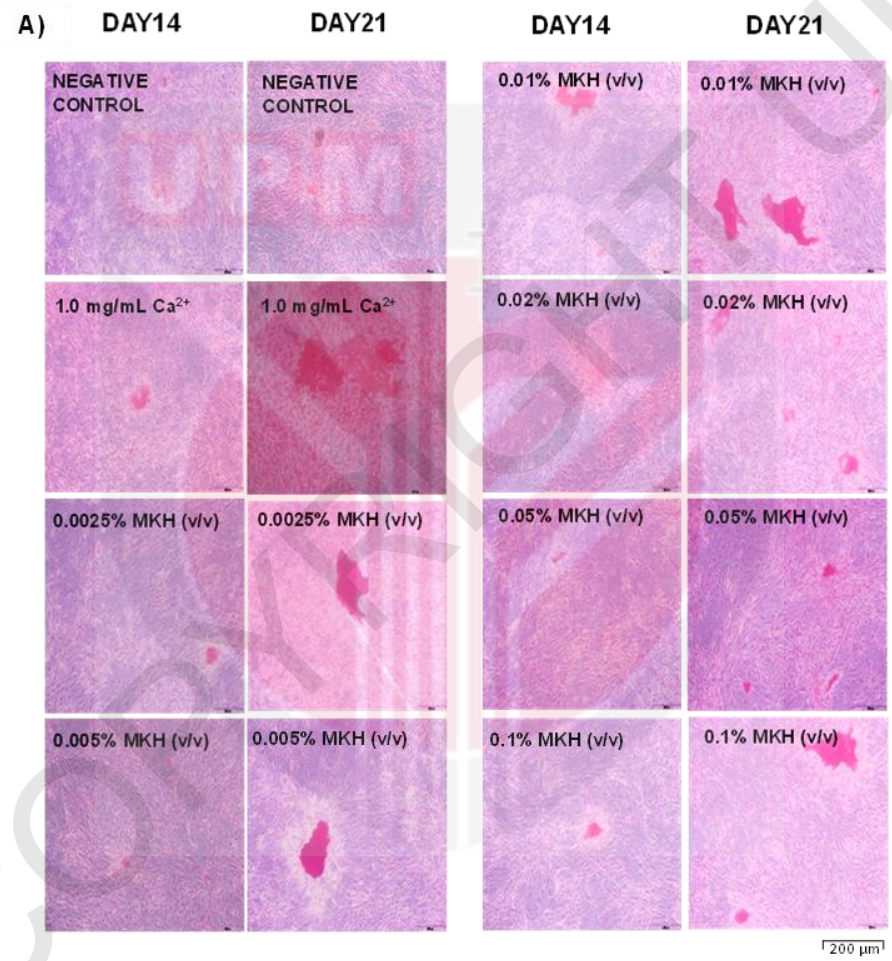


Figure 4.5: Calcification of calcium formation in MC3T3-E1 cells after 14 days and 21 days. (A) representative images in size and intensity of the deep red spots were observed in various concentrations of MKH and 1.0 mg/mL calcium-treated groups of MC3T3-E1 (B) Absorbance at 570 nm in MC3T3-E1 cells for quantification by acetic acid for 14 days and 21 days. Data were expressed (n=3) as the mean absorbance ± SEM. ***p<0.001 were considered significant vs. their respective negative control at day 14 and 21. Multiple group comparisons between various concentrations of MKH were made at two-time points. #p<0.05 were considered significant.

In addition, the deposition of collagen was examined to assess the phenotypic differentiation of MC3T3-E1 cells. This qualitative assay, known as the Sirius Red staining assay, was performed at two-time points: day 14 and day 21. The assay aimed to evaluate the amount of collagen (mainly collagen type I and III fibres) deposited by osteoblasts, which contribute to the formation of dense and cross-linked collagen structures. Collagen comprises for approximately 85%-90% of the total organic bone matrix. The study demonstrated that as the size and intensity of the bright red clusters increased, the percentage of collagen content in the MC3T3-E1 cells also increased. This finding suggests a correlation between the visual observation of collagen deposition and the level of phenotypic differentiation in the cells.

According to Figure 4.6 (A), the deposition of bright red clusters was observed after the stain on day 14 of treatment and increased in size and intensity over time in all treated groups, including positive control and negative control groups by day 21. Other than that, the collagen content in MC3T3-E1 cells was quantified using a quantitative assay which was analysed colourimetrically. The absorbance values of bright red in MC3T3-E1 cells was proportional to the collagen deposition. In comparison between the same group, as shown in Figure

4.6 (B), significant differences were reported against their counterparts in all the treatment groups except for the cells treated with 0.1% v/v of MKH. The significant differences at ($p<0.05$) were observed in the positive control, 0.0025% v/v MKH, 0.005% v/v MKH, 0.01% v/v MKH and 0.02% v/v MKH when compared to their respective counterparts between the two time-intervals. Besides that, the negative control and 0.05% v/v MKH reported significant differences at ($p<0.01$) and ($p<0.001$), respectively, when compared to their respective groups on day 14.



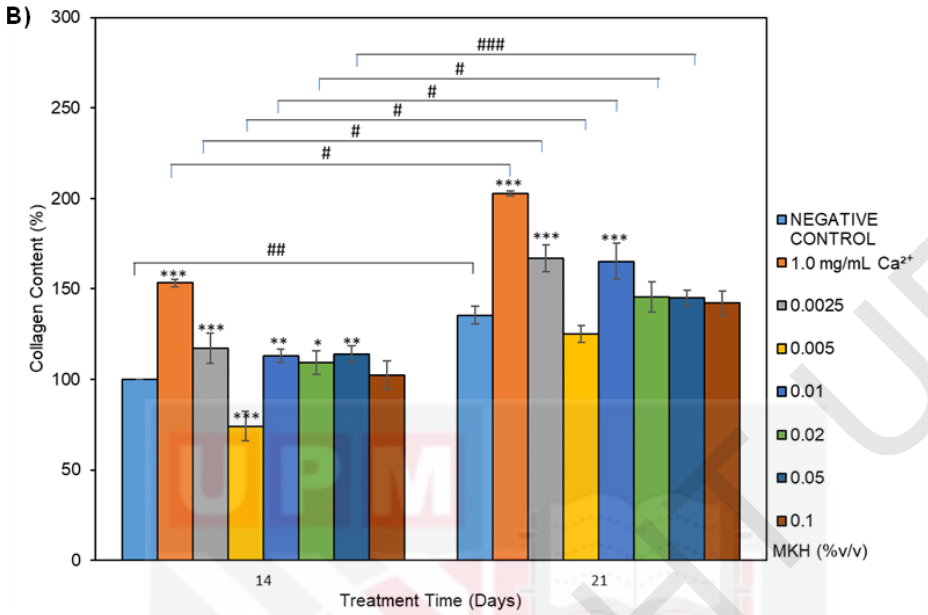


Figure 4.6: Calcification of collagen formation in MC3T3-E1 cells after 14 days and 21 days. (A) representative images in size and intensity of the bright red spots were observed in various concentrations of MKH and 1.0 mg/mL calcium-treated groups of MC3T3-E1. (B) Percentage collagen in MC3T3-E1 cells for quantification by diluting hydrochloric acid for 14 days and 21 days. Data were expressed (n=3) as the mean absorbance ± SEM. *p<0.05, *p<0.01 and ***p<0.001 were considered significant vs. their respective negative control at day 14 and 21. Multiple group comparisons between various concentrations of MKH were made at two-time points. $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 were considered significant.

In this study, the positive control group deposited the highest percentage of collagen content compared to all treatment groups after 14 days significantly ($p<0.001$) when compared to the negative control. Meanwhile, the percentage of collagen content was significantly ($p<0.05$) higher in the rest of MKH-treated groups of MC3T3-E1 cells when compared to the negative control except in the group treated with 0.005% v/v MKH, which was lower than the negative control group. The group treated with MKH at concentrations of 0.0025% v/v was significantly ($p<0.001$) higher compared to the negative control group. Beside that, 0.01% v/v and 0.05% v/v MKH-treated groups showed significant difference at ($p<0.01$), while group treated with 0.02% v/v MKH reported significant difference at ($p<0.05$) when compared to the negative control group.

The percentage of collagen content was significantly ($p<0.05$) higher on day 21 in the treatment groups, including both controls, compared to their counterparts on day 14. The positive control along with MKH-treated groups 0.0025% v/v MKH and 0.01% v/v MKH showed the higher percentage of collagen content with a significant difference ($p<0.001$) compared to the negative control on day 21.

Apart from that, MC3T3-E1 cells treated with 0.005% v/v MKH concentration reported the lowest percentage of collagen content compared to all treated groups and showed a significant difference ($p<0.05$) compared to the negative control group on day 21. Hence, the presence of treatment MKH exposed to the cells significantly enhanced the collagen density, as evidenced by the bright red clusters of collagens.

In addition to that, to investigate the impact of MKH on promoting inorganic-phosphate deposition in MC3T3-E1 cells, *von Kossa* staining was conducted at two time points: day 14 and day 21. The *von Kossa* staining method allows for the visualisation of dark brown spots, indicating the extent of inorganic-phosphate deposition. Inorganic-phosphate deposition plays a significant role in bone osteogenesis, and it is typically observed during the late stage of mineralisation.

Based on the photomicrograph results shown in Figure 4.7, the size and intensity of dark brown spots on day 14 in all groups were lower compared to their counterpart on day 21, including positive and negative controls. Thus, the size and intensity of the dark brown spots gradually increased in time until day 21.

On day 14 of treatment, the cells treated with calcium (positive control) showed the obvious deposition of dark brown spots compared to the negative control group. For MKH-treated groups, the concentration at 0.01% v/v MKH showed higher deposition of dark brown spots compared to the negative control. Meanwhile, on day 21 of treatment, the deposition of dark brown spots shown by positive control group was the highest compared to the negative control and the rest MKH-treated groups. As for the cells treated with MKH, 0.02% v/v promoted the deposition of dark brown spots compared to the negative control and the rest of the MKH-treated groups. Thus, the positive control and MKH at 0.02% v/v promoted the deposition of inorganic-phosphate in MC3T3-E1 cells during mineralisation.

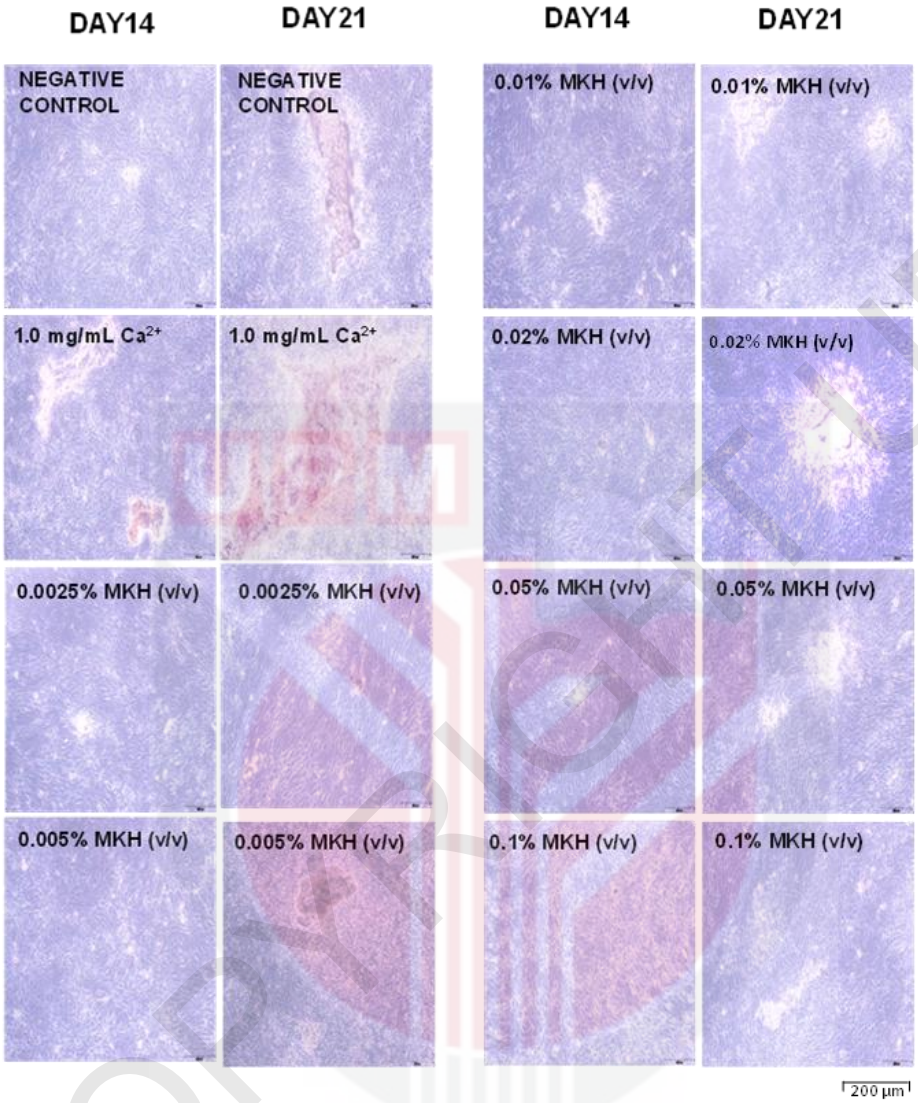


Figure 4.7: Calcification of inorganic phosphate in MC3T3-E1 cells after 14 days and 21 days. Representative images in size and intensity of the dark brown spots were observed in various concentrations of MKH and 1.0 mg/mL calcium-treated groups of MC3T3-E1**.**

The result showed the low MKH concentrations increased the colony, ALP activity, formation of calcium, collagen and inorganic phosphate during the differentiation phase in this study. This result coincided with Agasi et al. (2021), who reported that MKH could be maintained, increase the number of osteoblasts and encourage nodule formation in osteoblasts during the differentiation phase. Furthermore, the finding of the present study is in line with a previous study by Kamaruzzaman et al. (2019), in which MKH maintained and induced the replication rate of osteoblasts in glucocorticoid-induced rats by increasing bone

volume/tissue volume, trabecular number time-dependently and concentration-dependently during the differentiation phase after 2 months of treatment. The authors stated that MKH, which has antioxidants, becomes one of the factors that increase the differentiation activity of pre-osteoblasts and plays a role in the crosstalk of signalling pathways such as Wnt, bone morphogenic protein, Forkhead box protein O3, Runx2 and Osx that stimulated bone development (Kamaruzzaman et al., 2019; Ranneh et al., 2017).

Beside that, another review suggested that *tualang* honey could be used as an alternative to treat postmenstrual osteoporosis due to the presence of antioxidant properties against bone loss with minimal sides (Effendy et al., 2012). The result of this study was aligned with Yu et al. (2015), which found that *tualang* honey increased ALP activity after being added to HPDLFs for 7 days. The findings of this study coincided with the study by Zuhri et al. (2020), which found that the young males who consumed 20 g of *tualang* honey as a supplement and conducted physical exercise for 6 weeks were able to increase the secretion of collagen in their bodies. Moreover, the result of this study was supported by a review done by Ramli et al. (2021), who used different types of honey in which the daily consumption of *tualang* honey can exert the same effect on bone densitometry compared to the hormone replacement therapy. Honey consumption as a daily supplement also served as a good energy source and helped absorb calcium and magnesium retention. In addition, it contributes to stronger bones and teeth due to the presence of non-digestible carbohydrates such as raffinose, which produces short-chain fatty acid (SCFA) as the by-product of the fermentation process in the caecum and colon. The SCFA also helped lower the intestinal pH and created a favourable environment that increased minerals such as calcium solubility and absorption (Amin et al., 2018).

Besides that, honey contains various phenolic compounds such as kaempferol that benefit the human being (Olas, 2020). In the review by Wong et al. (2019), kaempferol isolated from *Polygonum tinctorium* increased ALP activity and calcification in murine pre-osteoblastic MC3T3-E1 cells. This finding was in line with the study by Xie et al. (2021), demonstrating that kaempferol, one of the phenolic compounds found in honey at 25 µM, induced the production of calcium in MC3T3-E1 cells when treated with that compound for 14 days. Besides, the current result also coincided with the study by Mazaki et al. (2013), which observed that 35 µM of kaempferol induced the production of inorganic phosphate in human mesenchymal stem cells (hMSC) derived from bone marrow when treated with kaempferol for 4 weeks. Furthermore, in their review, Wong et al. (2019) supported that kaempferol could also serve as a bone protecting agent because the presence of kaempferol increased the calcium content in the rat's femoral diaphyseal and metaphyseal tissue culture in their study. Apart from that, Gan et al. (2022) observed that 10um of kaempferol significantly increased the calcification and ALP activity after 2 weeks of treatments on bone marrow through mediation of SOX2/miR-124-3p/PI3K/Akt/mTOR[2] axis. Numerous studies have demonstrated that kaempferol

[2] SOX2 plays role in differentiation of osteoblasts and proliferative ability.

enhances osteogenesis differentiation through multiple pathways. The molecular mechanisms underlying its osteogenic potential have been extensively discussed, emphasising the possibility of phytochemicals like kaempferol being effective in addressing various bone disorders. Consequently, the presence of kaempferol in MKH (presumably referring to a specific substance or product) holds significant promise, as the study exhibited positive outcomes (Gan et al., 2022).

Other than kaempferol, flavonoid such pinocembrin, pinostrobin and vitexin found in honey. The study done by Natsume et al. (2021), which used pinocembrin in their study, which is the natural flavonoid from *Alpina zerumbeton*. Their result showed that 150 µM of pinocembrin was able to increase the ALP activity and OCN expression in MC3T3-E1 cells from day 3 and day 5 respectively after treatment during osteoblast differentiation. Furthermore, pinocembrin exhibited a protective effect against glucocorticoid-induced apoptosis in osteocytes by activating autophagy through the suppression of the PI3K/Akt/mTOR pathway. Osteocytes, the most prevalent cell type in bone constituting 90%-95% of bone cells, play a critical role in regulating the activities of osteoclasts and osteoblasts. Therefore, it is suggested that flavonoids in MKH may have the potential to promote osteoblast mineralisation (Wang et al., 2020). The result in this study correlated with the study by Gu et al. (2017), which used pinostrobin in their study and observed the formation of inorganic phosphate calcified nodules in MC3T3-E1 cells after being treated for 28 days with a maximum at 40 µg/mL in their study (Fahey & Stephenson, 2002; Gu et al., 2017). The presence of pinostrobin in different media could stimulate the deposition of inorganic phosphate time-dependent and be more potent than 17-β-oestradiol (Gu et al., 2017). Furthermore, Yuan et al. (2020) found that the presence of vitexin at the concentration of 10^{-8} µg/mL elevated the inorganic phosphate in the MC3T3-E1 cells after 28 days of treatment. Similar to pinocembrin, pinostrobin and kaempferol, vitexin is a phenolic acid that is present in a variety of plants and is also found in honey (Chew et al., 2018). Additionally, the study demonstrated that vitexin exhibited an *in vitro* anti-osteoporosis effect and might have some potential to treat glucocorticoid-induced osteoporosis (Yuan et al., 2020).

The osteoblasts in the osteogenic medium induced collagen formation with the extension of the exposure treatment time. It is important to note that the collagen content in this study directly correlated with cell mineralisation. The construction of highly dense hydroxyapatite microcrystals, necessary for creating a strong and compact bone composite, involves the removal of protons from the mineral through two linked transport steps. MKH was found to enhance collagen

miRNAs are regulating the cellular process via targeting mRNAs.
SOX2-miRNA regulatory network is known to play critical roles in cancer and inflammations.
PI3K/Akt/mTOR signalling is a crucial mediator in the cellular process.
Activation of PI3K/Akt/mTOR can lead to osteogenic differentiation in BMSCs.

deposition in osteoblasts and promote osteoblast differentiation, thereby improving osteoblast adhesion (Blair et al., 2017; Guo et al., 2017).

Besides that, during MC3T3-E1 cells mineralisation, the osteoblasts grew in the differentiated media in the presence of ascorbic acid, which played a vital function as a cofactor in the post-translational modification of collagen molecules and was thought to be the cause of the time-dependent increase in collagen formation. Furthermore, osteoblastic differentiation connects the collagen matrix (type I collagen) and cell surface integrin receptors (Thu et al., 2016). Therefore, MKH also has the potential to stimulate osteoblast maturation and differentiation as well as bone matrix creation, which was demonstrated by the increased magnitude of collagen deposition in the MKH-treated groups. Besides that, honey contains amino acids that are beneficial for synthesising other materials and energy sources, such as collagen, produced during synthesising structural proteins (Ummah et al., 2020). The study by Yolanda et al. (2021) showed that fibroblasts treated with honey for 7 days deposited higher collagen density than aloe vera during wound healing. As shown in the literature, faster maturation of collagen fibres in wound healing in the rat model was promoted by the presence of iron, copper, and ascorbic acid in honey (Yolanda et al., 2021).

In this study, Ca^{2+} concentration of 1.0 mg/mL was used as the positive control. The results demonstrated the effectiveness of the positive control in various assays, including colony formation, ALP activity, calcium, collagen, and inorganic phosphate deposition. The positive control showed significant difference ($p<0.001$) in colony formation, calcium, collagen and inorganic phosphate deposition as compared to the negative control on day 14 and day 21. The calcium-treated cells exhibited positive outcomes and demonstrated an expected increase, thereby validating the study's findings. The positive control in this study showed higher osteoblastogenesis compared to the negative control and MKH-treated groups, possibly due to calcium's ability to stimulate higher external and intracellular calcium concentrations. Calcium plays essential roles as a second messenger in various cellular processes and as a co-factor for enzymes in physiological functions like blood clotting. Osteoporosis involves reduced osteoblast activity and bone formation, making mineral supplementation to promote osteoblast proliferation beneficial for bone remodelling in this condition (Hirayama et al., 2013; Modi et al., 2019).

This study is supported by Hong et al. (2011), who claimed that the exposure of the cells to the calcium phosphate at the concentration of 4.52 mM for 9 hours could increase colony formation in human osteoblast-like cells (MG-63). Therefore, the presence of calcium in calcium phosphate in the treatment increases the number of osteoblasts, thus encouraging the osteoblasts to form colonies. Similarly, Kamba & Zakaria (2014) found that calcium treatment in the form of calcium carbonate ($CaCO_3$) caused higher ALP activity and calcium deposition in MC3T3-E1 cells compared to human foetal osteoblast (hFOB 1.19) cells after it was exposed to the treatment for 7 days. Furthermore, this finding is parallel with the study by Li et al. (2018), in which the observed $CaCO_3$

nanoparticles at 20 µg/mL stimulated the production of collagen on human bone marrow mesenchymal stem cells (hBMSCs) after 14 days of treatment.

Additionally, a study by Hirayama et al. (2013) observed increasing calcium and inorganic phosphate calcification in human dental pulp cells when treated with 10 µg/mL of $CaCO_3$ for 30 days. In summary, the study's positive control, along with supporting research, highlights the significant impact of calcium on various aspects of bone cell behaviour and osteoblast activity. Additionally, the research conducted by Al-Dujaili et al. (2016) demonstrated that the combination of calcium and parathyroid hormone (PTH) secretion increased the number of cells per bone volume and promoted the differentiation of bone marrow stromal cells (BMSCs). As a result, in the current study, the group treated with calcium outperformed the MKH-treated groups in colony formation during differentiation. Calcium balance in osteoblasts plays a vital role in maintaining bone formation and has been shown to be effective in supporting normal bone growth and development in children and adolescents, as well as in preserving bone mineral density in postmenopausal women (Vannucci et al., 2018).

4.2.2 Effects of MKH on osteocalcin (OCN) and osteoprotegerin (OPG) concentration in MC3T3-E1 cells

Osteocalcin (OCN) is one of the markers of the late differentiation of osteoblasts and, specifically, the main non-collagen extracellular matrix synthesised by osteoblasts (Choi et al., 2016; Jeong et al., 2021). It plays an important role in maintaining bone formation and inhibiting abnormal hydroxyapatite crystals. In addition to that, the production of osteoprotegerin (OPG) protein by osteoblasts and osteogenic stromal cells protects the bone from excessive resorption by binding to RANKL and blocking it from interacting with RANK. Hence, the effects of MKH-treated and calcium-treated in MC3T3-E1 cells on OCN and OPG concentration were investigated by using an enzyme-linked immunosorbent assay (ELISA) kit at two-time points namely days 14 and 21.

When compared between day 14 and day 21, the OCN concentration was found to increase significantly ($p<0.05$) in all treatment groups of MC3T3-E1 cells except for the negative control and 0.01% v/v MKH showed no significant differences ($p>0.05$) when compared to their respective groups as shown in Figure 4.8. Hence, this showed OCN concentration increased in the time-manner during mineralisation of MC3T3-E1 cells.

Based on the results in Figure 4.8, there was no significant observed on the OCN concentrations in all MKH-treated groups of MC3T3-E1 cells on day 14 although the OCN concentration of MKH-treated groups lower than the negative control except for the MC3T3-E1 cells treated with MKH at a concentration of 0.005% v/v which showed the lowest OCN concentration significantly ($p<0.05$) when compared to the negative control group. At day 14 of the treatment, the highest level of OCN was observed in positive control followed by negative control,

0.0025% v/v MKH, 0.01% MKH, 0.05% v/v MKH, 0.1% v/v MKH, 0.02% v/v MKH and 0.005% v/v MKH groups. However, on day 21, between all treated groups with negative controls, the differences were not significant (p>0.05).

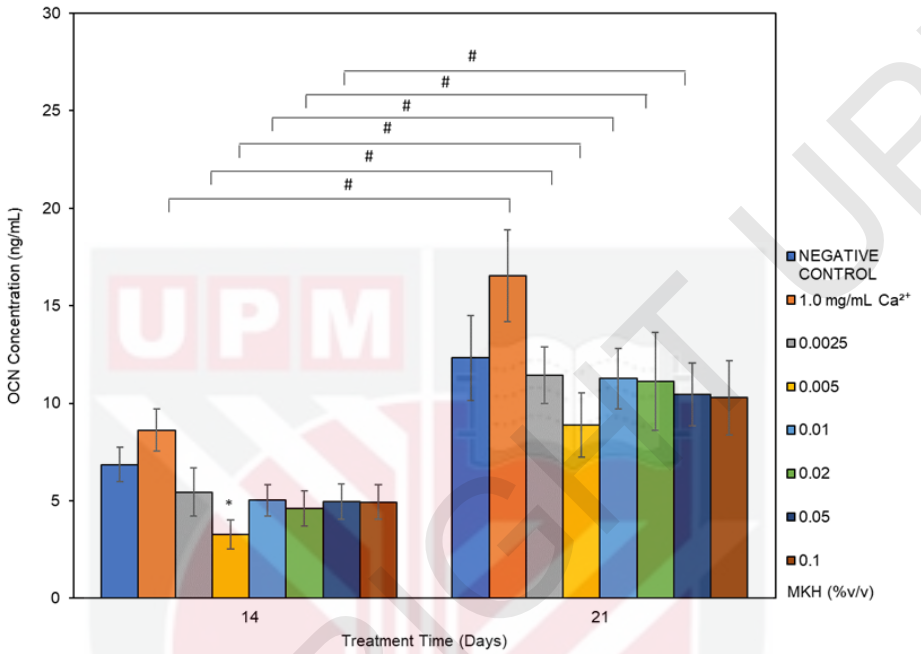


Figure 4.8: Effect of MKH on OCN concentration of MC3T3-E1 cells after 14 and 21 days by using OCN ELISA kit. Three independent experiments were carried out and data were expressed as mean of OCN concentration ± SEM. *p<0.05 was considered significant for treatment groups vs. their respective negative control at day 14. The comparison between days of treatment within the same groups was analysed by using student t-test. #p<0.05 was considered significant.

As shown in Figure 4.9, the result of OPG concentration on day 14 was significantly lower (p<0.05) in all treatment groups, including positive and negative control, compared to their counterparts on day 21. The negative control, groups treated with 0.0025% v/v MKH and 0.005%v/v MKH were observed significant difference at (p<0.05) against their respective groups, while the positive control (calcium-treated), 0.01% v/v MKH and 0.05% MKH showed significant difference at (p<0.01) when compared to their respective groups. Besides that, there were significant differences shown at (p<0.001) in the group treated with 0.02% v/v MKH and 0.1% v/v MKH compared to their respective groups.

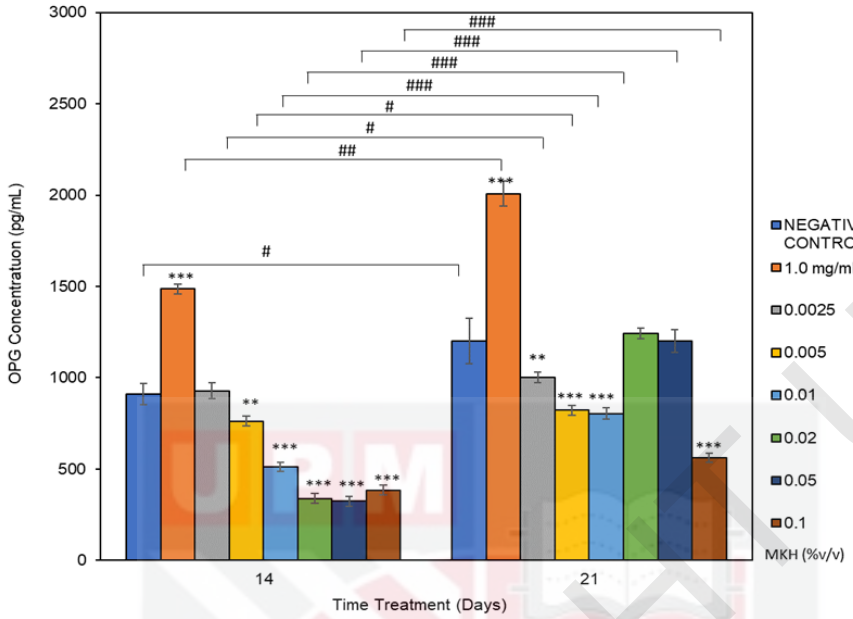


Figure 4.9: Effect of MKH on OPG concentration of MC3T3-E1 cells after 14 and 21 days by using OPG ELISA kit. Three independent experiments were carried out and data were expressed as the mean of OPG concentration ± SEM. **p<0.01 and ***p<0.001 were considered significant for treatment groups vs. their respective negative control at day 14 and day 21. The comparison between days of treatment within the same groups was analysed by using student t-test. #p<0.05, ##p<0.01, and ###p<0.001 were considered significant.

On day 14 of the treatment, the results showed that the OPG concentrations for the MKH-treated groups were significantly lower (p<0.01) except for the groups that were treated with 0.0025% v/v MKH when compared to the negative control. In contrast, the positive control group showed the highest concentration of OPG when compared to the negative control (p<0.001). Besides that, there was a significant difference (p<0.01) in the group treated with 0.005% v/v MKH compared to the OPG concentration against the negative control group. Meanwhile, lower OPG concentration was observed significantly (p<0.001) at 0.01% v/v MKH, 0.02% v/v MKH, 0.05% v/v MKH and 0.1% v/v MKH when compared to the negative control.

However, the OPG concentration steadily increased when measured on day 21 for all groups. The calcium-treated group showed the highest OPG concentration significantly (p<0.001) compared to the negative control on day 21. As for MKH-treated groups at 0.0025% v/v MKH, 0.005% v/v MKH and 0.1% v/v MKH showed significantly (p<0.01) lower compared to the negative control group. Beside that, there was no significant difference observed at 0.02% v/v and 0.05% v/v MKH-treated groups although OPG concentration reported higher than the negative control group.

The ability of osteocalcin (OCN) to bind calcium and hydroxyapatite correlates with the functionality of the organic matrix of the bone, dentin, and other mineralised tissues. Thus, it is required for hydroxyapatite binding and deposition in the bone ECM (Yodthong et al., 2018). Besides OCN, the expression of osteoprotegerin (OPG) is also important during the mineralisation phase. It binds to RANKL and acts as an anti-osteoclastogenesis factor. Osteoblasts commonly produce OPG beside other cells, such as the bone marrow. Therefore, the final development and activation of osteoclasts are inhibited by OPG and their apoptosis, which further regulates bone resorption. Besides, the upregulation of the OPG/RANKL ratio prevents osteoclastogenesis by binding to RANKL and inhibiting osteoclast differentiation (Hadjidakis & Androulakis, 2006; Nazrun et al., 2012; Tobeiha et al., 2020). Furthermore, recent research has also revealed that the increase in plasma OPG levels among postmenopausal women leads to increase bone mass, and the presence of OCN increases bone formation. Thus it can overcome osteoporosis (Moriishi et al., 2020; Tobeiha et al., 2020).

The increase in other bone-related protein biomarkers such as ALP, calcium, collagen and inorganic phosphate deposition also occurred alongside the increased OCN and OPG concentration. This study found that the level of OCN and OPG increased as the time of the cells treated with the treatment (MKH and calcium) increased in all treatment groups, including both positive and negative controls. Furthermore, the increase in OCN and OPG levels promoted bone mineralisation and inhibited bone resorption (Khotib et al., 2021).

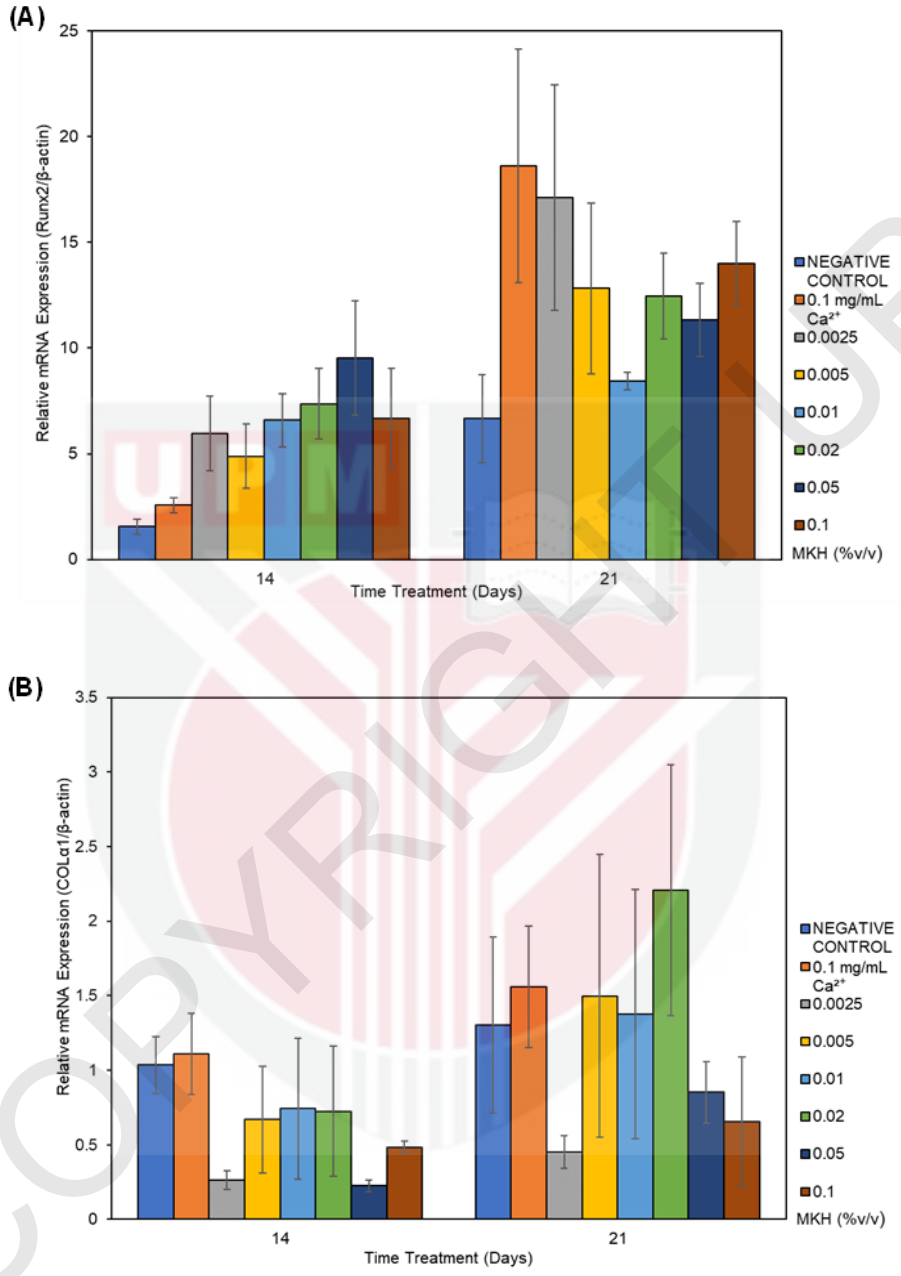
The ability of MKH to increase the level of OCN and OPG during mineralisation may be due to the presence of polyphenols in the MKH. Honey consists of polyphenols such as flavonoids and phenolic acids that work together to produce a range of synergistic antioxidant properties (Kamaruzzaman et al., 2019). Other than that, these findings were correlated with the study done by Jeong et al. (2021), who used rosmarinic acid as the treatment in their study. Rosmarinic acid is one of the total bioactive compounds found in honey (Gyergyák et al., 2016; Laaroussi et al., 2021). The study by Jeong et al. (2021) proved that 14 µg/mL of rosmarinic acid stimulated the production of OCN and OPG expression in MC3T3-E1 cells after being treated for 10 days due to the presence of a bioactive compound that contains antioxidant properties. Furthermore, during osteoblast differentiation, the antioxidant properties elevate the expression of ALP and Runx2 to stimulate osteoblast proliferation and reduce osteoclast production to facilitate bone reduction (Domazetovic et al., 2017). In addition, the treatment of MG-63 cell derived from a 14-year-old Caucasian male with caffeic acid, a phenolic compound present in honey, showed that 10^{-6} M of caffeic acid increased the OCN and OPG expression after 24 hours of treatment (Melguizo-Rodríguez et al., 2019; Olas, 2020).

In this study, it was found that calcium as positive control showed the highest OCN and OPG level in osteoblast cells compared to the MKH-treated groups. In addition, the expression of CoLα1 and OCN are also linked with the availability of calcium, which regulates the bone formation, metabolism and regeneration. Calcium is highly expressed in increasing skeletal tissue, which aids in bone

mineralisation and turnover processes (Modi et al., 2019). This finding was supported by Modi et al. (2019), who demonstrated in their study that treating MG-63 cells with 1 mM calcium glucoheptonate stimulated the OCN expression after being incubated for 48 hours. Besides that, the result was supported by Bae and Kim (2010), who employed calcium and supplemented magnesium as a treatment in their study that observed OPG expression level was increased in ovariectomised Sprague-Dawley female rats. According to Piri et al. (2016), calcium and other minerals such as magnesium, zinc and vitamin D injected subcutaneously 5 times a day for six weeks supplemented with oestradiol hormone increased the OPG levels and bone mineral density in newborn Wistar rats compared to the control. As a result, bone formation is increased as the OCN and OPG concentrations increases.

4.3 Effects of MKH on mRNA bone expression in MC3T3-E1 cells

To see if these findings could be applied to another protein expression found in the organic bone matrix, this study was continued by observing the expression of *Runt-related transcription factor 2* (Runx2), *collagen type 1 alpha-1 (*COLα1), and *bone sialoprotein* (BSP) mRNAs. Runx2 is a non-collagenous, highly conserved transcription factor in bone mineralisation matrix control. Runx2 mRNA expression entails a master regulator of osteogenic expression and bone formation that is upregulated in response to MKH treatment in cultured osteoblasts (Cho & Kwun, 2018; Zhou et al., 2015). Other than that, CoLα1 mRNA expression provides structural integrity for mineralised inorganic material deposition and plays a role in the mechanical resistance of bone tissue. Apart from that, *bone sialoprotein* (BSP) mRNA expression plays a key role in the initiation processes of bone matrix mineralisation and mineralised cell adhesion matrix formation through a specific signalling pathway (Khotib et al., 2021; Langenbach & Handsche, 2013).



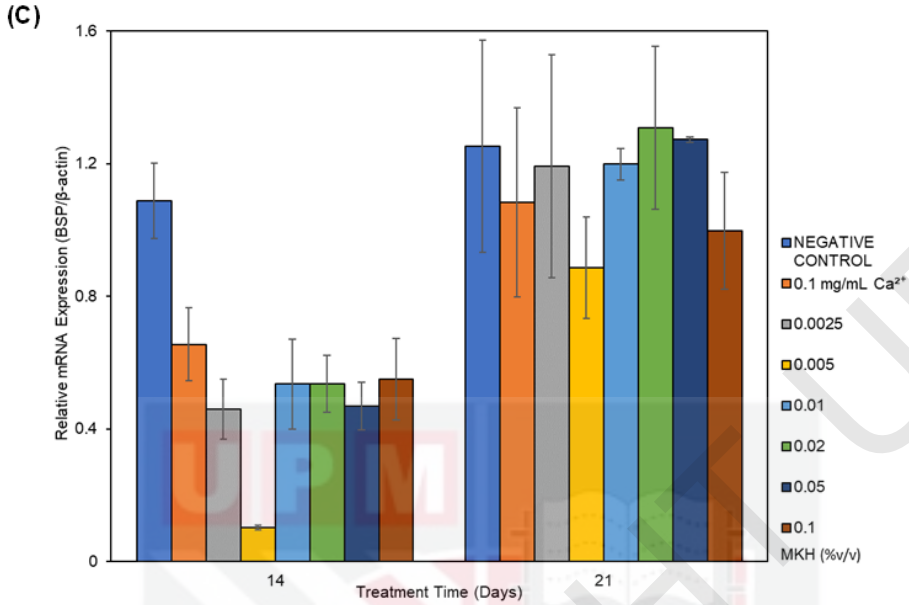


Figure 4.10: Effect of MKH on the bone specific mRNAs expressions in MC3T3-E1 cells after exposing MC3T3-E1 cells to various concentrations of MKH for 14 days and 21 days. (A) Runx2 (B) COLα1 (C) BSP mRNA expressions. Three independent experiments were carried out and data were expressed as mean ± SEM.

Therefore, the effects of MKH-treated and calcium-treated in MC3T3-E1 cells on Runx2 mRNA expression were investigated by qualitative reverse transcriptase polymerase chain reaction (qRT-PCR) at two-time points, namely day 14 and day 21. In this study, it was found that the bone-specific mRNAs expression, which is Runx2, CoLα1 and BSP, increased in a time-dependent manner to stimulate osteoblastic differentiation in all treatment groups, including both controls (positive and negative).

Based on the result of this study, the Runx2, CoLα1 and BSP mRNA expression in all treated groups of MC3T3-E1 cells, including a negative and positive controls, on day 21 were higher when compared to their counterparts on day 14, respectively as shown in Figure 4.10. However, there was no significant difference ($p>0.05$) was observed between two time-points for all bone specific mRNA expression. Based on Figure 4.10 (A), there was no significant shown in all treatment groups on day 14 compared to the respective control group, although the level of Runx2 mRNA expression in the MKH treated was higher. The highest level of Runx2 mRNA expression on day 14 was observed in MC3T3-E1 cells treated with MKH at concentrations of 0.05% v/v followed by 0.01% v/v, 0.1% v/v, 0.02% v/v, 0.0025% v/v, 0.005% v/v, positive control and negative control groups.

Moreover, on day 21 showed no significant difference shown in MKH treated groups compared to the negative control, but the Runx2 mRNA expression was higher in MKH treated group compared to the control. The calcium-treated showed the highest Runx2 mRNA expression compared to the rest and proved the positive control was worked well Note that the highest level of Runx2 mRNA expression on day 21 was observed in groups treated with calcium (positive control) followed by groups treated with 0.0025% v/v MKH, 0.005% v/v MKH, 0.1% v/v MKH, 0.02% v/v MKH, 0.05% v/v MKH, 0.01% v/v MKH and negative control.

Figure 4.10 (B) showed the result of COLα1 mRNA expression. There was no significant difference when compared respectively to their counterparts on day 21. However, the COLα1 mRNA expression of MKH treated groups were lower compared to the negative control. The highest level of COLα1 mRNA expression on day 14 was observed in positive control group followed by groups treated with MKH at concentrations of 0.02% v/v, 0.01% v/v, 0.02% v/v, 0.005% v/v, 0.1% v/v, 0.0025% v/v and 0.05% v/v.

However, as the treatment time increased, the presence of COLα1 mRNA expression steadily increased, although there were no significant observed when compared to day 21 for all treatment groups. The comparison between all treated groups and negative control showed no significant difference ($p>0.05$) in COLα1 mRNA expression on day 21. The highest level of COLα1 mRNA expression on day 21 was observed in groups treated with MKH at 0.02% v/v followed by positive control, 0.005% v/v MKH, 0.01% v/v MKH, negative control, 0.05% v/v MKH, 0.1% v/v MKH and 0.0025% v/v MKH.

Based on the result in Figure 4.10 (C), there were no significant different shown on day 14 in all treatment group for BSP mRNA expression on day 14 when compared respectively to their counterparts on day 21. The level of BSP mRNA expression on day 14 in MKH treated groups observed no significant difference although were lower compared to negative control. The highest level of BSP expression on day 14 was observed in untreated MC3T3-E1 cells (negative control) followed by positive control, 0.01% v/v MKH, 0.1% v/v MKH, 0.02% v/v MKH, 0.0025% v/v MKH, 0.05% v/v MKH and 0.005% v/v MKH-treated groups.

Same as day 14, there was no significant difference ($p>0.05$) in BSP mRNA expression shown on day 21 between all treated groups compared to the negative control. Moreover, the BSP mRNA expression of all treated groups was lower than the negative control. The highest level of BSP expression on day 21 was observed in untreated MC3T3-E1 cells (negative control) followed by the group treated with 0.02% v/v MKH, 0.01% v/v MKH, 0.05% v/v MKH, 0.1% v/v MKH, 0.0025% v/v MKH, positive control and 0.005% v/v MKH.

The expression of bone-specific mRNAs is crucial during osteogenic differentiation, which plays an important role in the maturation and stabilisation

of osteoblasts. Runx2 mRNA expression entails a master regulator of osteogenic expression and bone formation that is upregulated in response to MKH treatment in cultured osteoblasts (Cho & Kwun, 2018; Zhou et al., 2015). Other than that, CoLα1 mRNA expression provides structural integrity for mineralised inorganic material deposition and plays a role in the mechanical resistance of bone tissue. Apart from that, *bone sialoprotein* (BSP) mRNA expression plays a key role in the initiation processes of bone matrix mineralisation and mineralised cell adhesion matrix formation through a specific signalling pathway (Khotib et al., 2021; Langenbach & Handsche, 2013). In this study, it was found that the bone-specific mRNAs expression, which is Runx2, CoLα1 and BSP has no significant difference on MKH-treated cells.

Kaempferol, pinocembrin and quercetin are the polyphenols commonly found in honey, promoting osteoblasts' differentiation and mineralisation (Mandal & Jaganathan, 2009; Olas, 2020). Apart from that, the presence of these polyphenols in honey increased the mRNAs expression of Runx2, CoLα1 and BSP in the osteogenic differentiation of osteoblasts through bone morphogenetic protein (BMP), extracellular signal-regulated kinases (ERK) and p38 signalling pathway (Natsume et al., 2021; Yang et al., 2010; Zhou et al., 2015). The trend of the current result was in line with the findings reported by Kim et al. (2016), in which the Runx2 and CoLα1 mRNA expression increased in MC3T3-E1 cells when exposed to 10 μM of kaempferol for 24 hours. Besides that, Yang et al. (2010) evidenced that 5 μM of kaempferol elevated the secretion of BSP mRNA expression during osteoblast mineralisation in osteoblast-like (UMR 106) cells isolated from rat osteosarcoma after 12 hours of treatment and Runx2 mRNA expression after 6 hours treatment. Furthermore, another study by Natsume et al. (2021) has also evidenced that 150 μM pinocembrin isolated from *Alpinia zemrubet* induced the Runx2 mRNA expression level in MC3T3-E1 cells after 24 hours of exposure.

Furthermore, the result of this study was supported by Zhou et al. (2015), who used quercetin, the polyphenols commonly found in honey, the same as rosmarinic acid (Kamaruzzaman et al., 2019). The finding in this study was supported by Zhou et al. (2015) showed that the quercetin at a concentration of 2 μM stimulated Runx2, CoLα1 and BSP mRNA expression levels in BMSCs isolated from a 4-week-old male after being treated for 24 hours. This is further supported by Shi et al. (2022), who demonstrated that quercetin promotes proliferation, differentiation and mineralisation in the osteoblastic lineage with a concomitant increase in the expression of osteogenic genes, while the concentration of quercetin needed for proliferation and differentiation may be cell type-dependent.

Similar to MKH-treated cells, the bone-specific mRNA expression in the calcium-treated group increased in a time-dependent manner from day 14 to day 21 in this study. This was supported by Wang et al. (2011) also found that calcium hydroxide at 0.4 mM stimulated the expression of Runx2 and BSP mRNA in human osteoblast-like cells (Saos2) at 3 hours, and the effect became maximal at 12 hours. In another study by Wang et al. (2018), they demonstrated that

calcium hydroxide at a concentration of 0.4 mM enhanced the BSP mRNA expression level in rat osteosarcoma-derived osteoblast-like (ROS 17/2.8) cells and stromal bone marrow cells after 12 hours of exposure.

Besides that, the increasing bone-specific mRNA expression in the calcium-treated group is supported by Fei et al. (2012). They found that the expression of the Runx2 mRNA expression was enhanced by the extracts of calcium silicate starting from day 3. It became more evident with the increase in the amount of calcium silicate (100%) upon exposure to osteoblast-like cells isolated from the calvaria of neonatal Sprague-Dawley rats after being treated for 10 days. Furthermore, Jia et al. (2015) revealed in their study that the highest Runx2 mRNA expression was observed in the primary renal tubular epithelial cells after being treated with Ca^{2+} at a concentration of 10^{-8} mol/L for 48 hours of treatment. This finding agreed with Modi et al. (2019), who found that calcium glucoheptonate at 1.0 mM elevated CoLα1 mRNA expression on MG-63 cells after being treated for 7 days. Bone formation, metabolism and regeneration are all regulated by the CoLα1 mRNA expression and OCN concentration, which directly correlate with calcium availability. Thus, increasing the bone-specific mRNA expression level in time-manner demonstrated that the calcium treatment mineralised the osteoblasts during the mineralisation phase.

CHAPTER 5

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Conclusion

From the outcomes of this study, it could be concluded that Malaysian *Kelulut* Honey (MKH) at low concentrations increased the viability and the proliferation of MC3T3-E1 cells. However, higher concentrations of MKH reduced the viability and proliferation of this cell line.

The result of the phenotypic differentiation in MC3T3-E1 cells was summarised in the Table 5.1. The result showed that the ALP activity and deposition of collagen significantly increased in MKH-treated cells when compared to the negative control. apart from ALP activity and deposition of collagen, the colony formation, collagen formation and osteoprotegerin (OPG) concentration in MKH-treated cells also increased although not significant. Beside that, the inorganic-phosphate deposition was similar as negative control and the concentration level of osteocalcin (OCN) lower compared to the negative control. However, the treatment with positive control (1.0 mg/mL of calcium) was able to increase the phenotypic differentiation of MC3T3-E1 cells better than MKH.

Table 5.1: Summary result of the phenotypic differentiation in MC3T3-E1 cells

Name of Assay	MKH vs –ve control	MKH vs +ve control
Colony forming assay	Increased (not significant)	Decreased
ALP assay	Increased (significant)	Increased
Alizarin Red assay	Increased (not significant)	Decreased
Sirius Red assay	Increased (significant)	Decreased
von Kossa assay	Same	Decreased
ELISA OCN assay	Decreased	Decreased
ELISA OPG assay	Increased (not significant)	Decreased

Furthermore, the result in Table 5.2 showed that the expression of osteoblastic differentiation mRNAs, namely *Runt-related transcription factor 2* (Runx2), *collagen type 1 alpha-1* (COLα1) and *bone sialoprotein* (BSP) in MC3T3-E1 cells in MC3T3-E1 cells were comparable to the negative control. However, the

treatment with positive control (0.1 mg/mL of calcium) was able to increase the osteoblasts mineralisation of MC3T3-E1 cells better than MKH.

Table 5.2: Summary result of the expression of osteoblastic differentiation mRNAs in MC3T3-E1 cells

mRNAs expression	MKH vs –ve control	MKH vs +ve control
Runx2	Increased (not significant)	Increased
COLα1	Increased (not significant)	Increased
BSP	Decreased (not significant)	Decreased

In conclusion, this study indicates that MKH stimulated the viability and proliferation of MC3T3-E1 cells, but at high concentrations, it exhibited inhibitory effects. Additionally, MKH treatment resulted in positive outcomes in ALP activity and collagen deposition in the osteogenic condition for MC3T3-E1 cells. However, the mechanism of MKH towards osteoblastic differentiation and mineralisation remains unclear and it might not have a definitive role in osteoblastic differentiation in MC3T3-E1 cells. Nevertheless, the future research in needed for more comprehensive outcomes.

5.2 Limitations of the research

As this study suggested that MKH may have the potential in promoting bone health, the results obtained were confined to several limitations. The limitation of this study was conducted only on one type of bone cell line, namely murine preosteoblasts cell lines (MC3T3-E1). Osteoblasts are the cells that are responsible for bone formation. Because of that, this study could not represent the whole bone mechanism process. Other than that, this study was only conducted *in vitro,* thus the findings were limited to the cells cultured in controlled environments. Due to that, the findings may not reflect the effect of MKH on the whole body system.

Besides that, this study only used MKH obtained from a few sources. As the chemical composition in honey affected by geography and climate (Badrulhisham et al., 2020), the findings from this study could not be used to conclude the general effects of MKH on MC3T3-E1 cells. Honey from different sources may give different results on this cell line. Apart from that, this study only focused on the effects of MKH on the MC3T3-E1 cells but not the other types of honey. Different types of honey such as *tualang* may give different results on this cell line due to the different chemical composition in the honey. In addition to that, time constraint was the one of the limitations in this study. The differentiation and mineralisation process of the MC3T3-E1 cells to become mature osteoblasts

is long taking up approximately 20 days (Liu et al., 2021). Thus, the effect of MKH on MC3T3-E1 cells may be different if the period of the study is extended.

5.3 Recommendations for future research

To fulfill the limitations of research stated above, the researcher suggests that to further explore the effect and mechanism of MKH on bone cells, the researcher suggests that this study could be expanded by using other types of bone cell lines such as osteoclasts and osteocytes. Osteoclasts are responsible with bone resorption, whereas osteocytes serve as mechanosensors and orchestrators of the process of bone remodelling (Florencio-Silva et al., 2015). By studying the effect of MKH on the other types of cells, the bigger picture of MKH on the bone health could be obtained. MKH may not as effective as calcium in promoting osteoblastic differentiation of MC3T3-E1 cells, however it may possess the inhibition effect on osteoclastogenesis as osteoclasts reduced bone resorption activity which subsequently maintained the bone health (Effendy et al., 2012).

Besides that, to further explore the effect of MKH on bone cells, the researcher suggests the study could be conducted *in vivo.* The study conducted on *in vivo* is more specific and reliable for observing biological effects in body system. Hence, the overall efficacy of MKH on bone health can be investigated.

To further investigate the effect of MKH on bone cells, the researcher suggests varieties of MKH from different regions in Malaysia to be used. This is due to difference chemical composition of MKH affected by geography and climate and will give different results. Hence, the overall effect of MKH in Malaysia towards the bone health has a clearer explanation.

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APPENDICES

Appendix A

Authentication certificate of MC3T3-E1 cells

2018/01/09

RIKEN BRC CELL BANK

Resource No.	RCB1126
Resource Name	MC3T3-E1
Lot No.	65
General character	Differentiate to osteoblasts. Collagen producing.
Explain	骨芽細胞に分化して骨を作る細胞株。コラーゲンを比較的大量に産生する。
Class for catalog	d
Depositor	Kodama, Hiroaki
Originator	Kodama, Hiroaki
Year of deposit	1994
Original cell	MC3T3-E1
Animal	mouse
Strain name	C57BL/6
Gender	Unknown
Age at sampling	0 day
Tissue derived	newborn, calvaria
Classification	other
Lifespan	infinite
Morphology	fibroblast-like
Anchordepd	Adherent cells
Medium and additives	MEM ALPHA + 10% FBS
Antibiotics	Free
Pasmethod	0.25% trypsin
Passage ratio	1 : 4 split
SC frequency	Subculture : 2 times/week
Temperature	37℃
C02 concentration	5%
Freeze medium	Medium + 10% DMSO
Passage No.	U+7
Freezconc	1.0x10^(6) (cells/tube)
Viability at thawing	70%
Adhesion efficiency	>100%
Mycoplasma	(−)
Isozyme	Lot.40 : LD, NP

以下余白

Appendix B

MC3T3-E1 cells without any treatment

BIODATA OF STUDENT

Nur Najihah binti Ahmad Puat was born in Hospital Parit Buntar, Perak on September 21, 1991. She received her early education at Sekolah Kebangsaan Sungai Bogak in Perak (1998-2003) before preceding to Sekolah Menengah Kebangsaan Agama (SMKA) Kerian in Gunung Semanggol, Perak (2004-2006) then transfer to Sekolah Berasrama Penuh Integrasi (SBPI) Gopeng in Gopeng, Perak (2007-2008). In February 2015, she was graduated with second upper class Bachelor of Science (Honours) In Engineering (Biochemical-Biotechnology) from International Islamic University Malaysia (IIUM), Gombak.

PUBLICATION