POTENTIAL OF STINGLESS BEE HONEY IN MODULATING SKIN AGEING OF HUMAN DERMAL FIBROBLAST CELLS

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POTENTIAL OF STINGLESS BEE HONEY IN MODULATING SKIN AGEING OF HUMAN DERMAL FIBROBLAST CELLS

by

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LIST OF SYMBOLS

α	Alpha
°C	Degree of Celsius
%	Percentage
µg	Microgram
µM	Micromolar
ml	Milliliter
min	Minutes
µl	Microliter
CO_2	Carbon Dioxide
rpm	Revolutions per Minute
s	Second
v/v	Volume per Volume

LIST OF ABBREVIATIONS

COL1A1	Collagen Type I
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
IPS	Institut Pengajian Siswazah
MEM	Minimum Essential Medium
MMP	Matrix Metalloproteinase
DNA	Deoxyribonucleic Acid
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SD	Standard Deviation
USM	Universiti Sains Malaysia
UV	Ultraviolet

POTENSI MADU KELULUT DALAM MEMODULASI PENUAAN KULIT PADA SEL FIBROBLAS KULIT MANUSIA

ABSTRAK

Kulit merupakan indikasi nyata bagi penuaan. Semasa penuaan, peningkatan penghasilan spesies oksigen reaktif (ROS) menyumbang kepada peningkatan penghasilan matrik metalloproteinase (MMP) yang menyebabkan pemecahan kolagen. Peningkatan penghasilan ROS boleh dihalang oleh tindakan antioksidan seperti madu kelulut. Madu kelulut merupakan sumber antioksidan semula jadi yang berpotensi melambatkan proses penuaan kulit. Oleh itu, tujuan kajian ini dijalankan adalah untuk mengkaji potensi madu kelulut dalam memodulasi proses penuaan kulit sel fibroblas manusia. Kepekatan optimum dan tempoh inkubasi madu kelulut ditentukan dengan menggunakan asai MTS, manakala peroksidasi lipid dianalisis menggunakan sitometer aliran. Ekspresi gen matrik metalloproteinase 1 (MMP-1) dan kolagen jenis I (COL1A1) dianalisis menggunakan teknik tindak balas rantai polimerase masa nyata. Hasil kajian menunjukkan bahawa kepekatan madu kelulut pada 0.02% dalam tempoh inkubasi selama 72 jam telah meningkatkan viabiliti sel fibroblas manusia berbanding dengan sel kawalan. Perlakuan dengan 0.02% madu kelulut selama 72 jam juga menunjukkan peningkatan bererti terhadap ekpresi gen kolagen jenis I (COL1A1) di dalam sel fibroblas senesen dan penurunan bererti terhadap ekspresi gen matrik metalloproteinase 1 (MMP-1) di dalam sel fibroblas pra-senesen dan sel fibroblas senesen. Walau bagaimanapun, tiada perubahan bererti bagi analisis peroksidasi lipid di dalam sel fibroblas pra-senesen dan sel fibroblas senesen yang diperlakukan dengan madu kelulut. Kesimpulannya, kajian ini mencadangkan bahawa madu kelulut berpotensi memperlahankan proses penuaan

kulit melalui modulasi ekspresi gen dengan menunjukkan penurunan ekspresi gen MMP-1 dan berpotensi meningkatkan sintesis kolagen melalui peningkatan ekpresi gen COL1A1 di dalam sel fibroblas manusia.

POTENTIAL OF STINGLESS BEE HONEY IN MODULATING SKIN AGEING OF HUMAN DERMAL FIBROBLAST CELLS

ABSTRACT

Skin is the visible indicator of ageing. During ageing, the excessive production of reactive oxygen species (ROS) contributes to the increasing of matrix metalloproteinase (MMP) production that causes collagen degradation. Increasing formation of ROS can be prevented by antioxidants such as stingless bee honey. Stingless bee honey is a good source of natural antioxidants that might delay skin ageing. Therefore, this study aims to elucidate the potential roles of stingless bee honey on the modulation of skin ageing in human dermal fibroblast cells. The optimum concentration and incubation time of stingless bee honey were determined using MTS assay, while lipid peroxidation analysis was quantitated using flow cytometer. Gene expression of matrix metalloproteinase 1 (MMP-1) and collagen type I (COL1A1) were analysed using real time RT-PCR. Results showed that the concentration of stingless bee honey at 0.02% for 72 hours incubation significantly increased the viability of human dermal fibroblast cells compared to untreated cells. Treatment with 0.02% of stingless bee honey for 72 hours also showed significant upregulation of COL1A1 expression in senescent human dermal fibroblast cells and downregulation of MMP-1 expression in both pre-senescent and senescent human dermal fibroblast cells. However, no significant changes were observed on lipid peroxidation analysis in both pre-senescent and senescent human dermal fibroblast cells when treated with stingless bee honey. In conclusion, this study suggested that stingless bee honey beneficially delayed skin ageing through downregulation of

MMP-1 expression and potentially promotes collagen synthesis through upregulation of COL1A1 expression in human dermal fibroblast cells.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Skin appears as a visible indicator of age. Structurally, the skin has two layers, dermis and epidermis that are attached via basal lamina (Rinnerthaler et al., 2015). The skin protects the body from the loss of fluids and the influx of microbial agents and chemicals (Moravvej et al., 2009). Skin is also involved in the body's homeostasis and defence mechanism towards microorganisms (Moravvej et al., 2009).

Skin ageing can be defined as an alteration of the skin functions and structure. Dry skin, wrinkle, pigmentation, and reduction of the tensile strength are the features of skin ageing (Sjerobabski-Masnec & Šitum, 2010). The alteration of the dermal stroma that mainly consists of extracellular matrix and fibroblast leads to skin ageing (Parrinello et al., 2005; Waldera-Lupa et al., 2014). Fibroblast cells can regenerate quickly and involved actively in the formation of collagen, elastin and glycosaminoglycan, which are the components of the extracellular matrix (Kurniawati et al., 2015; Yoshino et al., 2016).

During skin ageing, the quality and quantity of collagen production by the fibroblast in dermal skin reduced due to exposure to the sun and UV radiation (Tiedtke & Marks, 2007). The collagen produced by fibroblast is important for healthy skin (Tiedtke & Marks, 2007). It is the most abundant structural protein in human skin which can be degraded by the activity of matrix metalloproteinase (MMP) (Egeblad & Werb, 2002). MMPs or matrixins are zinc-dependent endopeptidases that function to degrade the components of the basement membrane

and extracellular matrix (Loffek et al., 2011; Benson et al., 2013). According to DeHeven (2014), skin sustains about 80% of free radical damage from exposure to the sun which usually occurs during active metabolic turnover.

Reactive oxygen species (ROS) can be produced and degraded by the aerobic organism. In the required condition, reactive oxygen species (ROS) are involved in various biological processes and act as messengers in cell signalling (Yang et al., 2018). The generation of a low level of ROS is required to activate the proliferation pathway for stem cell proliferation (Schieber & Chandel, 2014). However, an excessive amount of ROS can lead to oxidative stress (Poljsak & Dahmane, 2012). In inflammatory regulation, although ROS is important as a second messenger in the innate and adaptive immune cell, a high level of ROS within the immune cell can result in tissue damage (Schieber & Chandel, 2014). ROS also can affect cellular macromolecules and induced genomic instability and mutations. However, genetic instability often elicits additional ROS, which triggers cell senescence and apoptosis, thus acting as anticarcinogenic by limiting the further proliferation of the transformed cells (Milkovic et al., 2019). Besides, excessive ROS level also can cause skin ageing through matrix metalloproteinase (MMP) activation which triggered collagen degradation and affect the structure of the extracellular matrix (ECM) (Kammeyer & Luiten, 2015).

Besides regulates the MMPs expression, ROS molecules are also involved in enhancing lipid peroxidation (Rinnerthaler et al., 2015). During ageing process, lipid peroxidation reduces the integrity of the cell membrane (Stojiljković et al., 2014). Polar lipid is part of the lipid bilayer of the cell membrane components. It involves the production of subcellular organelle and cell's permeable barrier (Ayala et al., 2014). As the membrane lipid peroxidation increases, it can evoke inflammatory and

immune response, activate cell proliferation, gene expression and initiates apoptosis (Briganti & Picardo, 2003). Membrane protein damage and DNA mutation caused by lipid peroxidation contribute to the changes in the function and structure of the skin.

Human skin is equipped with antioxidants as protection against ROS (Kammeyer & Luiten, 2015). Skin antioxidant is needed to defend the cells from oxidative injury and oxidation products (Briganti & Picardo, 2003). During ageing, the antioxidative defense system against ROS is suppressed (Choi et al., 2012). Elevated ROS that leads to MMP-1 activation can be inhibited by antioxidants (Varani et al., 2006). Antioxidants treatment can increase the resistance of an organism to ROS and prevents skin ageing (Xu et al., 2018).

Stingless bee (*Apidae: Meliponini*) is widely distributed in all tropical and subtropical regions except in a few oceanic islands (Salim et al., 2012). Stingless bee honey has been widely used as an antioxidant because it has greater medicinal values than other types of honey (Shahjahan et al., 2007). Since stingless bee honey is well known to have antioxidant properties, therefore the study on stingless bee honey as one of the potential natural resources of anti-ageing needs to be explored on human skin.

1.2 Objectives of the study

1.2.1 General objective

To determine the effect of stingless bee honey in modulating skin ageing of human dermal fibroblast cells.

1.2.2 Specific objectives

- **Objective 1**

  To determine the optimum concentration and incubation time of stingless bee honey in human dermal fibroblast cells.

- **Objective 2**

  To determine the effect of stingless bee honey on lipid peroxidation and morphology of human dermal fibroblast cells.

- **Objective 3**

  To determine the effect of stingless bee honey on gene expression of matrix metalloproteinase 1 (MMP-1) and collagen type I (COL1A1) mRNA in human dermal fibroblast cells.

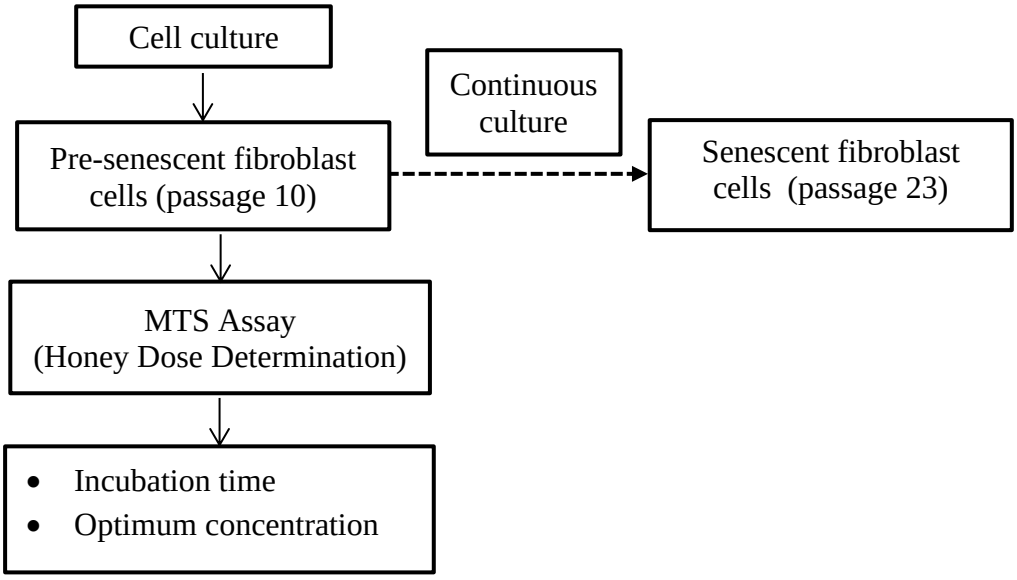
1.3 Research hypothesis

1. Stingless bee honey significantly reduces lipid peroxidation during ageing of human dermal fibroblast cells.
2. Stingless bee honey significantly reduces MMP-1 mRNA expression and promotes collagen synthesis during ageing of human dermal fibroblast cells.

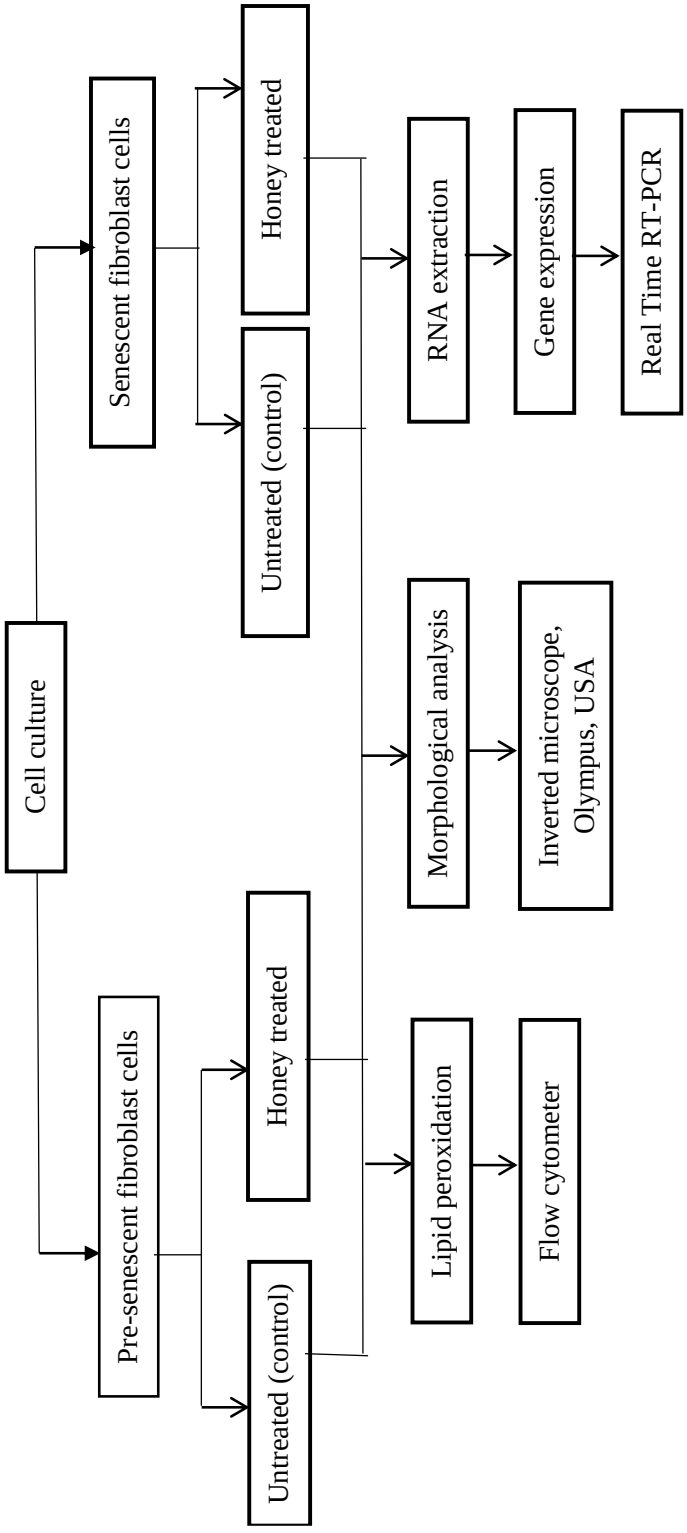
1.4 Flowchart of the study

The flowchart below shows the overview of this research.

(A)



(B)


Cell culture
Senescent fibroblast cells
Honey treated
Untreated (control)
Pre-senescent fibroblast cells
Honey treated
Untreated (control)
RNA extraction
Gene expression
Real Time RT-PCR
Morphological analysis
Inverted microscope, Olympus, USA
Lipid peroxidation
Flow cytometer

CHAPTER 2

LITERATURE REVIEW

2.1 Theory of Ageing

Ageing happened as we grew older. As ageing occurred in all living organisms, research on ageing increased over the year (Hohn et al., 2017). Ageing is an irreversible process due to the accumulation of physiological and morphological changes throughout the body (Akila et al., 2007). As we age the changes in the development of age-related diseases and death increase (Vina et al., 2007). The process of ageing has been explained in many proposed theories of ageing that classify two main categories, which are programmed theory and damage theory of ageing (Sergiev et al., 2015).

The programmed theory explained ageing as time-dependent changes in the regulation of growth and development including changes in defence system and gene expression (Jin, 2010). During ageing, a weakened immune system gives rise to infection and auto-immune diseases (Vina et al., 2007). Meanwhile, the damage theory of ageing suggested that environmental factors able to induce cellular damage during ageing (Jin, 2010). Accumulation of cellular deterioration being the risk factor for cancer, neurodegenerative disease, diabetes, and cardiovascular disorders (Lopez-Otin et al., 2013). Ageing also can be explained by the free radical theory created by Denham Harman, which proposed that ageing is the result of oxidative damage toward the cells and tissues of an organism (Gladyshev, 2014; Harman, 1955). During ageing, the endogenous antioxidant in our body unable to counterbalance the free radicals which are continuously generated by our body (Vina et al., 2007). *In vitro* study, showed more production of reactive oxygen species (ROS) and accumulation of

oxidized protein, lipid, and DNA during mitochondrial electron transport chain (Vina et al., 2007).

2.1.1 Factors of Ageing

Ageing is divided into two categories which are intrinsic ageing and extrinsic ageing that caused by both intrinsic and extrinsic factors (Kammeyer & Luiten, 2015). Both types of ageing showed different characteristics (Janson et al., 2013).

Intrinsic ageing referred to ageing that occurred along with time. It includes changes in genetics, metabolic process and cellular metabolism (Ganceviciene et al., 2012). As time passes by, our body accumulates endogenous damage due to the formation of reactive oxygen species (ROS) from cellular metabolism (Puizina-Ivic, 2008). Whereas, extrinsic ageing is caused by external stimuli such as cigarette smoke, ultraviolet (UV) radiation, environmental pollution, and poor nutrition (Puizina-Ivic, 2008). About 80% of the extrinsic ageing is contributed by the UV radiation which triggered the formation of ROS (Poljsak & Dahmane, 2012). There are two types of UV radiation; UVB and UVA which penetrated the epidermis of the skin, however only UVA can penetrate deeply into the human dermis resulting in the changes of dermal connective tissue (Puizina-Iviv, 2008). Degeneration of elastic fiber, reduction of collagen, and collagen type I and II precursor are the changes that developed in the dermal extracellular matrix during photoaging (Sjerobabski-Masnec & Šitum, 2010).

2.1.2 Skin ageing

Skin appears as a visible marker of ageing (Farage et al., 2013). Skin is the largest body organ that acts as a barrier system, covering all the underlying soft tissues (Nilforoushzadeh et al., 2017). Figure 2.1 showed that skin is composed of three connecting layers (epidermis, dermis, and subcutaneous tissues) that have different properties, nature, and functions (Escoffier et al., 1989). The component of the skin is compromised by the fibroblast cells which produce the extracellular matrix components such as elastin fibers and collagen (Makpol et al., 2011). The outermost layer of the skin, the epidermis composed of keratinocytes that synthesize keratin (Kolarsick et al., 2011). Meanwhile, the middle layer is the fibrous dermis mostly made up of collagen for about 80% of dermal skin dry mass (Uitto, 2008). The subcutaneous tissue contains lipocytes, a small lobe of fat cells that specialized in energy storage (Kolarsick et al., 2011).

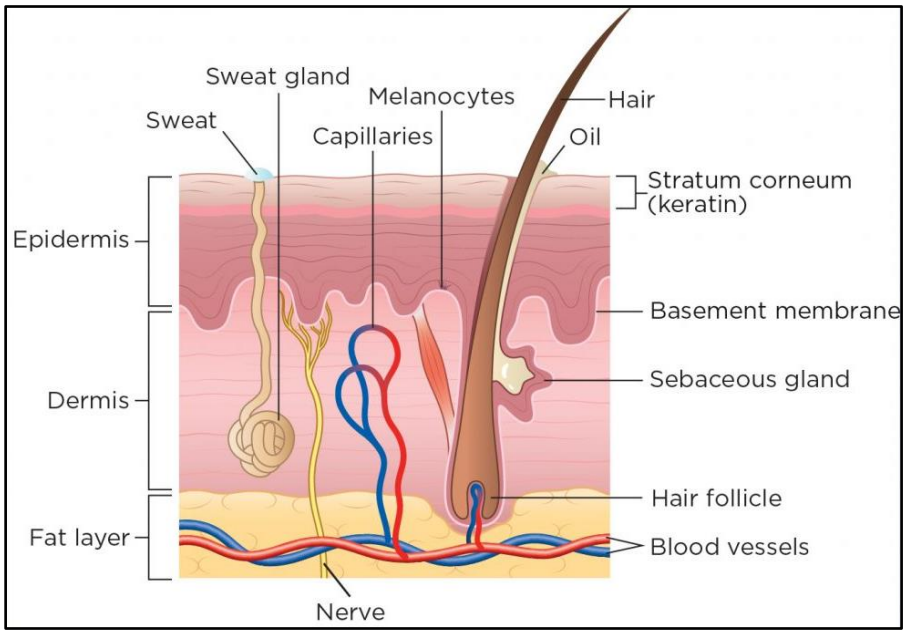


Figure 2.1 Skin layers (Lawton, 2019)

Skin serves as a barrier between the external environment and the internal tissue of the body (Farage et al., 2013). It is highly selective on what is moving in and out of the body (Ng & Lau, 2015). Skin plays important role in the protection against water loss, bacterial infection, injuries, and temperature adjustment (Nilforoushzadeh et al., 2017). Besides, the dermis of the skin provides tensile strength, pliability, and elasticity (Kolarsick et al., 2011). During ageing, skin function reduces and the risk for dermatological disorders and skin diseases increases (Farage et al., 2013).

As we become older our skin shows various visible and obvious signs of ageing (Zhang & Duan, 2018). In aged skin, the thickness of the dermis decrease as well as the number of fibroblast and mast cells (Farage et al., 2013). Elastin and collagen are the dermal connective tissue associated with ageing. During ageing, the elastin fibers degrade, collagen cross-link accumulates, and the collagen bundles are disorganized (Farage et al., 2007). A decreasing number of fibroblasts cause the collagen turn-over in aged skin to reduce (Farage et al., 2007). The interconnecting network of collagen fibrils and elastin that provides support to the epidermis and contributes to the skin elasticity and resilience can be altered during ageing (Zhang et al., 2020). Hence, the outcome of ageing can be predominantly observed in the skin as sagging, dry and wrinkling skin (Jenkins, 2002). Puizina-Ivic (2008), stated that the appearance of the wrinkle in the skin is the result of muscle changes, facial and cartilage substance loss, gravitational forces and losing subcutaneous fat tissue.

The rate of ageing in the skin can be determined when tissue degeneration exceeding tissue regeneration (Kammeyer & Luiten, 2015). In aged skin, skin losses its barrier function and the rate of wound healing decreases (Janson et al., 2013). A previous study by Escoffier et al., (1989) showed that skin able to maintain its

extensibility and thickness up to the seventh decade and reduced its recovery capacity and elasticity along with ageing.

2.2 Cellular senescence

Cellular senescence occurred when cells undergo a stable cell cycle arrest which is recognized when the cells were unable to replicate or lose their proliferative capacity (Herranz & Gil, 2018). As senescence occurred, cells started to undergo characteristic changes known as senescence markers (Hohn et al., 2017). Hohn et al., (2017) stated that changes in morphology, increased in senescence-associated β-galactosidase (SA-β-Gal) activity, protein modification, decreased turnover metabolism, proliferation arrest, accumulation of protein aggregates and formation of senescence-associated secretory phenotypes (SASP) were features of senescent cells.

The development of cellular senescence is the contributor to ageing (Hohn et al., 2017). Hayflick and Moorhead (1961) described the link between ageing and senescence through the limited capacity of cultured human fibroblast proliferation. This is also known as the Hayflick limit that explained the shortened telomere during the replication process. The Hayflick system of serial cultivation of diploid cell has been used widely to study ageing (Joergensen & Rattan, 2014). *In vitro,* a cell population able to age as the percentage of senescent cells during the culture is progressively increased until they reach the growth-arrested state in which the cells no longer dividing (de Magalhaes, 2004). The cells were no longer dividing and reach replicative senescence due to the shortening of telomere and stress response mechanism to proteolytic insult by trypsinization activity in each subcultivation of the cells (de Magalhaes, 2004). Cellular senescence can be studied by using different types of ageing models included *in vitro* aged cells which obtained from both aged

and young donor, stress-induced premature senescence (SIPS) model and aggregate-fed cell model (Hohn et al., 2017).

2.2.1 Fibroblast as a cellular ageing model

Fibroblasts are the most abundant cells in the connective tissue which were derived from mesenchyme (Nilforoushzadeh et al., 2017). Fibroblast is spindle-shaped cells that build up the major components of the skin dermis (Makpol et al., 2011). Fibroblast cells can migrate to the substrate layer (Nilforoushzadeh et al., 2017). Fibroblast cells regenerate quickly and serve in the formation of collagen, elastin and glycosaminoglycan, which are the components of the extracellular matrix (Kurniawati et al., 2015; Yoshino et al., 2016). It acts as an important precursor in the secretion of extracellular matrix components, involves in the generation of the skin structure and initiates cellular signaling (Nilforoushzadeh et al., 2017).

A decrease in cell growth rate and morphological changes arose from the aged human dermal fibroblast cells (Lago & Puzzi, 2019). Previous research showed the number of fibroblast cells reduced due to the reduction of the proliferation activity of fibroblast cells during ageing (Gunin et al., 2011). Aged fibroblast cells exhibit a larger size of cells compared to the young cells (Lago & Puzzi, 2019).

Human dermal fibroblasts are the best model system to study cell physiology (Giampieri et al., 2018). Serial passaging was used as *in vitro* model of ageing due to its effect on primary cells and expressed the hallmarks of cellular senescence (Janson et al., 2013). Normal human cells have a limited capacity to proliferate in culture (Cristofalo et al., 2000). The proliferative potential of normal cells in culture is the result of multiple changes and the development of specific senescence markers when

it became senescence (Hohn et al., 2017). A study by Hayflick & Moorhead (1961) showed that in culture, fibroblast has limited proliferation capacity.

2.3 Hallmarks of cellular senescence

Specific hallmarks could determine the rate of age in an individual (Dodig et al., 2019). These hallmarks of ageing can be grouped into three categories that are primary hallmarks, antagonistic hallmarks and integrative hallmarks (Aunan et al., 2016). The primary hallmarks include genomic instability, telomere attrition and epigenetic alteration (Aunan et al., 2016). The stability of genetic materials such as DNA can be disturbed by the exogenous agents and endogenous threats leading to the accumulation of genetic damage and lesion (Lopez-Otin et al., 2013). In the chromosome, telomeres act as an indicator of the ability of the cells to replicate (Dodig et al., 2019). Cells have limited proliferative capacity as the telomere becomes shorter in every replication process, thus causing ageing (Lopez-Otin et al., 2013).

Antagonistic hallmarks of ageing refer to the response of our body towards damage such as loss of proteostasis, deregulated nutrient sensing, and mitochondrial dysfunction (Aunan et al., 2016). Protein homeostasis is important in maintaining the function of the protein. Cellular damage produced during the normal ageing process might interrupt the maintenance of proteostasis resulting in a misfolded protein that promotes the development of age-related diseases (Lopez-Otin et al., 2013). Mitochondria is the source of energy as well as producing free radicals through the electron transport chain. However, the integrity of the mitochondrial reduced during ageing resulting in more production of oxidative stress that causes harm to our body (Aunan et al., 2016).

Meanwhile, the integrative hallmarks of ageing are specified by cellular senescence and altered intracellular communication that cause changes in the phenotype (Aunan et al., 2016). Cellular senescence occurs as the cells undergo stable cell cycle arrest and no longer dividing (Hohn et al., 2017). As the cells deteriorate during ageing, the communication between the cellular components is altered. In the older organism, the increase of inflammation response is implicated in the failure of the immune system and accumulation of tissue damage (Lopez-Otin et al., 2013).

2.4 Changes of extracellular matrix genes during ageing

The extracellular matrix (ECM) is the core substance composed in the dermis of the skin (Yagi & Yonei, 2018). The extracellular matrix is composed of protein, water and polysaccharides which provides a scaffolding effect towards the cellular components and essential in some biochemical cues such as homeostasis, tissue differentiation and morphogenesis (Frantz et al., 2010). The dermal ECM is mainly composed of fibronectin, collagen type I and III, proteoglycans and elastin (Puizina-Ivic, 2008).

During ageing, extracellular matrix proteins such as collagen and elastin are reduced (Uitto et al., 2008). Among the features of skin ageing is the decreasing collagen productions caused by the activity of matrix metalloproteinase 1 (MMP-1) (Graf et al., 2015). Collagen fibers are disorganized, shortened and thinned in photoaged skin (Fligiel et al., 2003). Degradation of extracellular matrix and reduction in collagen synthesis demonstrated the main sign of skin ageing at the molecular level (Yoshino et al., 2016). During ageing, cells unable to bind properly with the extracellular matrix as the arrangement of collagen fiber are altered (Sprenger et al.,

2010). The unbalance of matrix synthesis and breakdown during ageing results in skin ageing features such as wrinkles (Kular et al., 2014).

2.5 Genes involved in extracellular matrix degradation

2.5.1 Matrix metalloproteinase

Matrix metalloproteinase (MMP) is part of the zinc and calcium endopeptidase family known as proteinase which responsible for the degradation of the extracellular matrix (ECM) components (Visse & Nagase, 2003). The MMPs are then classified as Table 2.1 based on their specificity towards the basement membrane (Egeblad & Werb, 2002). Since MMPs responsible for tissue destructions due to its proteolytic activity, it is rigidly controlled under normal condition (Kim & Joh, 2012).

Table 2.1 Classification of MMP

Enzyme	**MMP**
Collagenase	1, 8, 13
Gelatinase	2, 9
Stromelysins	3, 10
Membrane type MMP	14, 15, 16, 17, 24, 25
Matrilysins	7, 11, 26
Others	12, 19, 20, 21, 23, 27, 28

MMPs are secreted as inactive zymogen and need to be activated by a serine protease, cleavage of NH2-terminal or activated by other members of the family (Rundhaugh, 2003). The activation of MMP by the other members in the family have been shown in the previous study towards the activation of MMP-9 by cleavage of

proMMP-9 by stromelysin-1or MMP-3 (Ogata et al., 1992). MMPs can be expressed by many different cells. For example, in the skin, MMPs were expressed by fibroblast, keratinocytes, endothelial cells and immune cells such as macrophages and monocytes (Caley et al., 2015). As MMPs are divided into various groups, they are also regulated differently (Benson et al., 2013). MMPs have been involved in many physiological and pathological processes such as cell proliferation and differentiation, cell migration, tissue repair, angiogenesis, chemokine inactivation, apoptosis, metastasis, fibrosis, and ageing (Sardy, 2009).

In the skin, collagen type I is the most abundant structural protein (Fisher et al., 2009). As we aged, the accumulation of fragmented collagen fibrils increased, thus impairs the properties of the skin and function of the cell in the dermis (Fisher et al., 2009). The breakdown of collagen is regulated by the activity of MMPs and tissue inhibitors of matrix metalloproteinases (Zhang et al., 2017). MMP-1 or collagenase is the class of MMP that accountable for the degradation of collagen since it initiates the cleavage of fibrillar collagen type I and fibrillar collagen type III (Zhang et al., 2017; Benson et al., 2013).

During ageing, the damage of skin connective tissue is mediated by the elevated action of multiples MMPs in the dermis (Quan & Fisher, 2015). A previous study in sun-protected skin showed that the fibroblast expresses a higher level of MMP-1 in aged human skin compared to young (Fisher et al., 2009). Besides, Brennan et al., (2003) found that MMP-1 is the major collagenase responsible for collagen destruction in photoaging skin compared to MMP-13. The presence of antioxidant might be partially reverse the activity of MMPs during ageing. In photoaging skin, the high level of MMP-1 and MMP-2 have been reduced with the

treatment of marigold methanol extract which possesses antioxidant activity (Kang et al., 2018). The previous study also had demonstrated that the antioxidative effect of rice wine able to reduce UV-induced matrix metalloproteinase-1 (MMP-1) expression in the cultured human fibroblast (Seo et al., 2009).

2.5.2 Collagen

The collagen family comprises 28 members (Table 2.2) and is numbered with Roman numerals (Ricard-Blum, 2011). They are classified into short-chain, basement membrane, fibrillar, or other class dependents on their function (Essays, 2018). The collagen is structured by the presence of triple helix with three polypeptide chain that varies according to its member (Ricard-Blum, 2011).

Table 2.2 Classification of collagen

	Type of collagen	**Tissue distribution**
Fibril-forming collagens	I	Bones, dermis, tendon, cornea, ligaments
	II	Cartilage, vitreous body, nucleus pulposus
	III	Skin, vessel wall, lungs, liver, spleen
	V	Lung, cornea, bone, fetal membranes
	XI	Cartilage, vitreous body
Basement-membrane collagen	IV	Basement membranes
Microfibrillar collagen	VI	Dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils	VII	Skin, dermal-epidermal junctions, oral mucosa, cervix

Table 2.2. Continued

	Type of collagen	Tissue distribution
Hexagonal network-forming collagens	VIII	Endothelial cells, Descemet's membrane
	X	Hypertrophic cartilage
Fibril-Associated Collagens with Interrupted Triple helices	IX	Cartilage, vitreous humor, cornea
	XII	Perichondrium, ligaments, tendon
	XIV	Dermis, tendon, vessel wall, placenta, lungs, liver
	XIX	Human rhabdomyosarcoma
	XX	Corneal epithelium, embryonic skin, sternal cartilage, tendon
	XXI	Blood vessel wall
Transmembrane collagens	XIII	Epidermis, hair follicle, liver endomysium, intestine, lungs chondrocytes
	XVII	Dermal-epidermal junctions
Multiplexins	XV	Fibroblasts, smooth muscle cells, kidney, pancreas
	XVI	Fibroblasts, amnion, keratinocytes
	XVIII	Lungs, liver

Note: Data are from Gelse et al., (2003).

Collagen is an essential protein in the skin as they are important for the extracellular matrix function and structure (Kular et al., 2014). Collagen reduces approximately 1% over the year and becomes disorganized and irregular in older skin (Ganceviciene et al., 2012). Ageing of cellular fibroblast and defect in mechanical stimulation of aged tissue cause the reduction of collagen synthesis (Varani et al.,

2006). The reduction of collagen synthesis also leads to a reduction of collagen turnover (Farage et al., 2013).

Collagen type I is the most structural protein in the human skin dermis and compromise about 90% of skin dry mass (Makpol et al., 2011). Collagen type I has a long biological lifespan and able to cause tissue dysfunction in the elderly as it undergoes several modifications on its properties over the time (Guilbert et al., 2016). Collagen type I is the best-studied collagen and had been studied extensively on skin ageing (Gelse et al., 2003). A study by Guilbert et al., (2016) revealed that collagen type I reduced in old-adult compared to young-adult and newborns. An *in vivo* study on skin ageing showed that the procollagen type I protein level in aged skin was reduced by 52% compared to the younger skin (Varani et al., 2000). The suppression of the expression of type I and type III procollagen in the dermis reduces the collagen content in the dermis (Kim & Park, 2016). Besides, *in vitro* ageing models using UVB irradiation and accelerated proliferation of human dermal fibroblasts from young and elderly donors showed a reduction of the expression of collagen type I in all models which revealed that gene expression was altered during ageing (Lago & Puzzi, 2019). Previous study also showed that the amount of collagen type I in young individuals was significantly higher than in old individuals (Bigot et al., 2012). The study demonstrated that during ageing process there were elevated amounts and binding activities of NF-κB, together with an increased number of senescent cells and ECM dysfunction which lead to senescence in dermal fibroblasts (Bigot et al., 2012).

2.6 Lipid Peroxidation

Lipid peroxidation has its role in maintaining cell permeability, cell proliferation and metabolism of membrane protein and lipid (Kisic et al., 2012). However, the imbalance between pro-oxidant and anti-oxidant lead to oxidative stress and give adverse effect to those processes (Kisic et al., 2012). A higher rate of lipid peroxidation could accelerate skin ageing due to molecular cell damage by apoptosis or programmed cell death (Ayala et al., 2014). An increase in oxidative damage to lipid, gene regulation, DNA and protein contributes to ageing (Rikans & Hornbrook, 1997). During ageing, the product of lipid peroxidation increase (Spiteller, 2001). Thus, an antioxidant is responsible to reduce lipid peroxidation, free radicals and DNA damage (Silva et al., 2017).

The human body has its endogenous antioxidant such as glutathione peroxidase (G-SH Px), superoxide dismutase (SOD) and glutathione (Akila et al., 2007). Oxidative stress caused by the exposure of UV radiation leads to lipid peroxidation, protein oxidation and differential expression of endogenous antioxidant enzymes (Lago & Puzzi, 2019). UV radiation (UVR) able to cause the generation of reactive oxygen species (ROS) and oxidative stress in human skin, when the formation exceeds the antioxidant defence of the target cells (Katiyar & Mukhtar, 2001; Poljsak & Dahmane, 2012). Reactive oxygen species (ROS) enhance the formation of reactive free radicals that able to give rise to various diseases and damage the biomolecules such as lipid, DNA and proteins (Drummen et al., 2002).

A previous study showed that in the elderly, the reduction of antioxidants triggers the level of lipid peroxidation (Akila et al., 2007). Lipid peroxidation occurs when ROS molecules attack the polyunsaturated fatty acid (Ayala et al., 2014). The

membrane protein damage and DNA mutation by lipid peroxidation can cause functional and structural changes in the skin (Stojiljković et al., 2014). The lipid peroxidation process can be induced by the changes in the structure of the cell membrane (Spiteller, 2001). Changes in the structure of the membrane lead to enzyme activation and give rise to oxidation products (Spiteller, 2001). Lipid peroxidation resulting in mutagenic and carcinogenic by-products such as malondialdehyde and 4-hydroxyl-2-noneal (Rinnerthaler et al., 2015). Malondialdehyde can induce functional and structural changes in the skin (Mason et al., 1997).

2.7 The role of antioxidant in ageing

Each organism is equipped with natural antioxidants to protect the body from oxidative stress. The antioxidant defence system in the body consists of both endogenous and exogenous antioxidants which able to scavenge and balance the free radicals (Seo et al., 2016). The endogenous antioxidant such as superoxide dismutase, glutathione, catalase and thiol peroxidase act as first-line antioxidant defence produced by the body (Hohn et al., 2017). In elderly people, the efficiency of endogenous antioxidative defence reduces and they become susceptible to oxidative stress (Tan et al., 2018). Furthermore, during high production of ROS, the antioxidative defence from endogenous antioxidants is inadequate, therefore the body required the presence of exogenous antioxidants such as vitamin E, vitamin C, trace elements, flavonoids, polyphenols and carotenoids (Aguilar, 2016; Hohn et al., 2017).

An antioxidant is important to balance the amount of oxidants in the body. Antioxidants prevent the oxidation of other molecules and cell damage by providing extra electrons to the free radicals (Okoduwa et al., 2015). A variety of antioxidants had been reported to protect against DNA damage and oxidative stress as well as

protecting the skin from photodamage. Despite scavenging ROS, supplementation with food rich in antioxidants increases cellular energy production and the immune system (Tan et al., 2018). The antioxidants also enhance metabolic rate and promote the longevity of a species (Vina et al., 2007). Antioxidant activities of honey had been widely demonstrated nowadays. Antioxidant activity and total phenolic compound of honey were proven to have a positive correlation and the antioxidant activity increased as the level of diluted honey increased (Al-Mamary et al., 2002).

2.8 Beneficial effect of honey

Honey is a beneficial natural product derived from both sting bees and stingless bees (Rao et al., 2016). The most common bees are European honeybees and stingless bees (Amin et al., 2018). European honeybees are *Apis Mellifera* which produced various types of honey such as Malaysia Tualang honey, jelly bush honey, African jungles honey and Manuka honey (Amin et al., 2018). Honey has been studied extensively all over the year and many reviews and research articles had been published on its benefits (Medhi et al., 2008). Honey has been used widely in modern and traditional medicine since it is produced by the nectar of a flower and composed of various compositions (Pathare et al., 2015). The quality of honey produced varies depending on the floral abundance, climatic and geographical abundance (Amin et al., 2018).

Honey is a saturated solution composed of various compounds such as fructose, glucose, amino acid, proteins, minerals, enzymes, vitamins and other minor bioactive compounds (Burlando & Cornara, 2013). Since honey is mainly composed of 80% to 85% of sugar, it is well known as a natural sweetener all around the world (Rao et al., 2016). Honey can be stored for a long time due to high osmotic pressure

and anti-bacterial properties which able to prevent the growth of microorganisms (Al-Mamary et al., 2002). Honey also has been used for a long time due to its therapeutic effects in both traditional and clinical usage (Burlando & Cornara, 2013).

Honey has been used widely for medicinal, industrial and nutritional purposes (Buba et al., 2013). According to traditional therapy, honey could relieve cough. Moreover, a previous survey study showed that a spoonful of honey was able to suppress cough in children (Ashkin & Mounsey, 2013). On the other hand, honey can reduce hair loss and dermatitis. A study by Al-Waili (2001) showed that skin lesions, itching, scaling and hair loss reduce after a few weeks of treatment with diluted crude honey.

Besides, honey has been proven to have antimicrobial activity. Honey plays its antimicrobial role by destroying the cell wall of the bacteria or inhibit metabolic pathways (Eteraf-Oskouei & Najafi, 2013). Previous research reported that honey has the inhibition potential toward 12 bacteria which are *Alcaligenes faecalis, Citrobacter freundii, Escherichia coli, Enterobacter aerogenes, Klebsiella pneumoniae, Mycobacterium phlei, Salmonella california, Salmonella enteritidis, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus and Staphylococcus epidermidis*. The greatest growth inhibition with score 3 (about 75% inhibition) was seen at 20% of honey treatment (Lusby et al., 2005). Apart from that, honey also can promote wound healing activities. Study showed that acacia and buckwheat honey participate effectively in the closure of the fibroblast wound (Ranzato et al., 2013). In addition, research by Sell et al., (2012) showed that Manuka honey can increase dermal regeneration by 90% healing area in three days. Honey also was tested clinically and showed 99% healing was completed within nine weeks with honey

treatment (Medhi et al., 2008). Furthermore, a study showed that Tualang Honey acts as an antioxidant in the pancreases of the streptozotocin-induced diabetic rats by reducing the level of elevated malondialdehyde (Erejuwa et al., 2010).

2.8.1 Stingless bee honey and its therapeutic effects

Stingless bee honey which is also known as Kelulut honey produced by the smallest bees without poisonous sting (Barakhbah, 2007). Stingless bee honey is different from other honey because the honey is produced in honey pots while the other honey is produced in the honeycomb (Saiful Yazan et al., 2016). Stingless bees are classified into genera Trigona and Melipona which produced honey known as 'Kelulut' honey, stingless bee honey and Meliponine honey (Amin et al., 2018). The composition of honey produced by Apis and stingless bees is different in viscosity, acidity, mineral and moisture content (Fatima et al., 2018). However, both produced similar nitrogen and ash content of honey (Fatima et al., 2018). Furthermore, stingless bee honey has been found to have higher total phenolic compound and color intensity than other honey such as Tualang Honey and Gelam Honey (Kek et al., 2014).

The therapeutic effect of honey depends on its microbiological, physical, and chemical characteristics (Amin et al., 2018). In traditional medicine, stingless bee honey has been used extensively to treat cough, bronchitis and cold (Amin et al., 2018). Stingless bee honey also was highlighted to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Shigella sonnei* and *Klebsiella pneumoniae* (de Queiroz Pimentel et al., 2013). Furthermore, Boorn et al., (2010) demonstrated the antibacterial activity of stingless bee honey by inhibition of *Staphylococcus aureus* through agar diffusion assay. Besides, a study by I1echie et al.,