BIOCHEMICAL AND MORPHOLOGICAL CHANGES OF UVB-IRRADIATED HUMAN SKIN FIBROBLAST (HSF1184) CELLS ON *Labisia pumila* EXTRACT

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Specially dedicated to:

My beloved husband..
Norman Hariss

My adorable newborn baby boy..
Ahmad Rafiqulhaq bin Norman (12th January 2015)

My dearest mom and dad, mother-in-law and father-in-law..
Lailah binti Ya’amat & Ab Karim bin Hasim
Jaminah binti Hashan & Hariss bin Ayob

My supportive family and in-law family members..

My mentors..
Dr. Harisun Yaakob & Prof. Dr. Mohamad Roji Sarmidi
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ABSTRACT

*Labisia pumila* var *alata* (LPva), or also known as Kacip Fatimah, is a traditional herb that has long been used to cure various ailments. Currently, it is reported to have a good anti-photoaging effect to the skin. However, an extensive research on this plant on anti-photoaging effect has yet to be studied in detail, especially in terms of the biochemical and morphological changes of human skin cells. Thus, the objective of this study is to determine the anti-photoaging effects of LPva plant extract on Ultraviolet B (UVB)-irradiated human skin fibroblast cells (HSF1184) and to observe the biochemical and morphological changes. In this study, HSF1184 cells were exposed to UVB irradiation and cultured in media without serum with LPva extract. After the UVB exposure, the changes in biochemical characteristics were investigated, which included cell viability, collagen synthesis, elastin synthesis and cell apoptosis. Finally, the morphological changes of the cells were evaluated using the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis. Result from cytotoxicity test showed that at concentration below than 0.0001 µg/mL, the LPva extract did not cause any toxicity effect to the HSF1184 cells, but at concentration higher than 10 µg/mL, the LPva reduced the viability of the cells. LPva extract was found to significantly increase the collagen and elastin synthesis, and also to decrease the apoptosis activity of the cells, as compared to the control. The microscopic observation through SEM indicated that at the highest concentration (1000 µg/mL) of LPva extract, all cells shrunk and died. Through TEM, this toxic concentration had caused inflammation to the mitochondria and it contained disorganized crystae, which indicated that the cells were programmed to death. Thus, this study revealed both biochemical and morphological changes on the UVB-irradiated HSF1184 cells after the treatment, and the results proved the anti-photoaging effect of LPva extract.
ABSTRAK

*Labisia pumila var alata* (LPva) atau dikenali juga sebagai Kacip Fatimah, adalah sejenis herba tradisional yang telah digunakan untuk mengubati pelbagai penyakit. Pada masa ini, ia dilaporkan mempunyai kesan anti-fotopenuaan yang baik untuk kulit. Walau bagaimanapun, penyelidikan secara menyeluruh tentang ekstrak tumbuhan ini terhadap anti-fotopenuaan belum dikaji secara terperinci terutamanya dari segi perubahan biokimia dan morfologi sel-sel kulit manusia. Oleh itu, objektif kajian ini adalah untuk menentukan kesan anti-fotopenuaan ekstrak tumbuhan LPva terhadap sel fibroblas kulit manusia (HSF1184) tersinar ultraungu B (UVB) dan untuk melihat perubahan biokimia dan morfologinya. Dalam kajian ini, sel-sel HSF1184 telah didedahkan kepada sinaran UVB dan dikultur dalam media tanpa serum dengan ekstrak LPva. Selonp pendedahan kepada UVB, perubahan pada biokimia telah dikaji melalui ujian daya maju sel, sintesis kolagen, sintesis elastin dan apoptosis sel. Akhir sekali, perubahan morfologi sel-sel tersebut dinilai dengan menggunakan mikroskop elektron pengimbas (SEM) dan mikroskop elektron pancaran (TEM). Keputusan ujian kesitotoksikan menunjukkan bahawa pada kepekatan kurang daripada 0.0001 µg/mL ekstrak LPva tidak memberi sebarang kesan ketoksikan kepada sel-sel HSF1184 tetapi pada kepekatan yang lebih tinggi daripada 10 µg/mL ekstrak LPva, sel-sel telah mati. Ekstrak Lpva didapati telah meningkatkan sintesis kolagen dan elastin dengan signifikan serta menurunkan aktiviti apoptosis berbanding dengan kawalan. Pemerhatian mikroskopik menerusi SEM telah menunjukkan bahawa pada kepekatan yang tertinggi (1000 µg/mL) ekstrak LPva, semua sel telah mengecut dan mati. Manakala melalui permerhatian TEM, kepekatan toksik ini telah menyebabkan keradangan pada mitokondria dan ia mengandungi susunan kristae yang tidak teratur menunjukkan sel-sel telah diprogramkan untuk mati. Oleh itu, kajian ini menunjukkan bahawa kedua-dua perubahan biokimia dan morfologi sel HSF1184 tersinar UVB selepas rawatan ini boleh digunakan untuk membuktikan kesan anti-fotopenuaan ekstrak LPva.
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CHAPTER 1

INTRODUCTION

1.1 Introduction

A huge number of Malaysian medicinal plants have long been used as traditional remedies from one generation to another up until today. The information on the plant materials that have been used as medicines since ancient times can be found in history books, old literature, pharmacopoeias and archaeological finds (Jantan, 2004). This fact is supported by the holy Quran and Bible, saying about 20 to 125 plants have been used as medicines to treat various diseases (Musselman, 1999). Out of more than 20,000 species of vascular plants in Malaysia, about 10%, or approximately 2,000 species, have been documented to have medicinal qualities (Herbal Medicine Research Centre, 2000). Thus, these have made most Malaysian nowadays seeking an option of consuming natural herbal remedies rather than synthetic medicines.

In line with these findings, Malaysian herbal product market is experiencing a tremendous growth. The natural products global trade was amounted to RM777 billion in 2006 and will be projected to triple to over RM2 trillion by the year 2020 (Economic Transformation Programme, 2011) as more people are turning to herbal
products as alternative to the conventional therapeutic medicine or as nutritional and dietary supplements (Jantan, 2004). However, there is concern for the lack of standardization of herbal preparations to guarantee their safety, quality and efficacy. Thus, there is an urgent need to conduct scientific research to provide experimental evidence of safety, efficacy and quality of herbal medicines, while also investigating the plants as sources for new lead structures for drug development (Jantan, 2004), especially the active compounds that are beneficial for cosmeceutical purpose.

In the 10th Malaysia Plan, the high value agriculture produces such as herbs and spices, organic fruits and vegetables, mushroom and seaweed will be given special focus to increase the contribution of Gross Domestic Product (GDP) to 2% by 2015 (Economic Planning Unit, 2010). Research and Development (R&D) grants will be given to research institutions based on research requirements requested by the participating companies. This effort is done with regard for the National Key Economic Area (NKEA) projects to embark the research program of some of the widely used medicinal plants, such as *Eurycoma longifolia* (Tongkat Ali), *Labisia pumila* (Kacip Fatimah), *Orthosiphon speciosa* (Misai Kucing), *Andrographis paniculata* (Hempedu Bumi) and *Phyllanthus niruri* (Dukung Anak) under close scientific and clinical scrutiny (Ministry of Agriculture, 2011). Currently, the Ministry of Agriculture had announced through the NKEA that all of these herbs are the top ten herbs in Malaysia that need to be fully utilized and developed as a vital source of the economy. Therefore, the ministry gives encouragement to the industries and researchers by providing a grant, covering from agronomy, processing, product development, standardization, processing and clinical trial. The changing trend towards the use of natural ingredients in product formulation is becoming very significant nowadays. Therefore it is hoped that the utilization of natural ingredients could be capitalized for mass production of herbal based cosmeceutical products.

*Labisia pumila* (*L. pumila*) or also known as Kacip Fatimah is one of the most popular herbal plants among Malay community which contain many bioactive compounds that are beneficial to human vitality. Recent study has shown that this plant contained several compounds include flavonoids, phenolic acids, benzoic acids
and also cinnamic acids (Chua et al., 2011). Flavonoids and phenolic acids have been proven containing antioxidant effect, which is very essential to promote healthy skin and provide skin protection from UV harm. As known, UV rays can enhance reactive oxygen species (ROS) generation in skin cells, which indirectly initiate the intracellular and extracellular oxidative stress; thus, they cause wrinkles and atypical pigmentation formation to the skin. These photo-induced aging skin symptoms can be prevented by practicing effective approach such as the use of antioxidants (Masaki, 2010). The same study by Chua et al. (2011) also reported that the presence of antioxidant activity in aqueous L. pumila extracts can help the skin against reactive oxygen species (ROS) by scavenging the free radical activity.

As ultraviolet (UV) radiation is a potent initiator of ROS generation in the skin, different UV wavelengths generate different types of ROS (Masaki, 2010). Ultraviolet B (UVB), for instance, stimulates the production of O$_2^-$ through the activation of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase and respiratory chain reactions (Jurkiewicz and Buettner, 1996), while ultraviolet A (UVA) generates $^1$O$_2$ through a photosensitizing reaction with internal chromophores, such as riboflavin and porphyrin (Masaki, 2010). UVA also produces ‘O$_2^-$ through NADPH oxidase activation (Valencia and Kochevar, 2008) and photosensitization of advanced glycation products (Masaki et al., 1999). However, $^1$O$_2$ is the major type of ROS produced on the skin surface, which is stimulated by a photosensitizing reaction with UVA and porphyrins from bacterial flora living on the skin (Ryu et al., 2009). $^1$O$_2$ is then oxidized to unsaturated acyl residues, cholesterol and squalene in the skin sebum to yield lipid hydroperoxides (Masaki, 2010).

The resulting oxidized lipids and proteins will then induce the alterations in skin conditions. Topical application of oxidized squalene (squalene monohydroperoxide) on the skin, for example, will disrupt the skin barrier function as an acute response and induce skin roughness as a chronic response (Chiba et al., 2003). Lipid hydroperoxides and proteins will be then further oxidized by alkyl aldehydes to produce stratum corneum carbonylated proteins (SCCP). Usually, the SCCP levels will increase following the UV exposure (Fujita et al., 2007) and during
winter season (Kobayashi et al., 2008). Patients who suffer from atopic dermatitis have higher levels of SCCP as compared to normal subjects (Niwa et al., 2003). The SCCP levels reflect the degree of oxidative stress in the skin induced by the environment, which is generally initiated by the UV-light. Thus, oxidative stress initiated by ROS can alter the skin conditions by accelerating skin aging and carcinogenesis (Getoff, 2007).

Oxidative stress that is initiated by ROS generation is an essential factor in modulating skin alterations, especially those caused by UV exposure and aging. The aging effect of excessive sun exposure on skin has been shown in the earliest recognition on the necks of sailors who suffer Cutis rhomboidalis nuchae, which includes the changes of textural and pigmentary of the skin when compared with the necks of nuns. Other consequence of chronic sun exposure is Favre-Racouchot disease or also known as modular elastosis, a condition where cysts and comedons form on the exposed parts of the skin (Giacomoni and Rein, 2004). Preliminary experiments with some antioxidants such as co-enzyme Q10, vitamin A and E, ferulic acid and silymarin which act as anti-aging compound, have been revealed able to repair long-term damage skin from environmental influences and also promote skin self-repair, which have been applied in topical skin care formulation (Pinnell, 2003). Thus, treatment using some antioxidants should be effective to enhance resistance to oxidative stress and improve skin aging (Masaki, 2010).

Recent study has demonstrated that all varieties of L. pumila plant extracts contain bioactive compounds that possess antioxidant activity (Karimi et al., 2011b). Besides, phytomolecules such as gallic acid contained in L. pumila extracts has been reported able to scavenge free radicals from skin cells, including a sun protector factor (SPF) of 15 or higher, and resulting in giving protection to the skin from wrinkles and leading to a healthy younger skin complexion (Mukherjee et al., 2011). Thus, this study was conducted to investigate the efficacy in term of biochemical and morphological changes when the L. pumila var. alata (LPva) plant extract, which acts as anti-photoaging agent, is applied on the UVB-irradiated Human Skin Fibroblast (HSF1184) cells.
1.2 Research Background

*Labisia pumila* (*L. pumila*) or also known as Kacip Fatimah among Malay community has been used as traditional remedy from generation to generation. It functions to ease and encourage the childbirth process, as well as to cure various ailments associated with women. There are three common varieties of *L. pumila* which can be found in Malaysia, which are *L. pumila* var. *alata*, *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata* (Stone, 1988). However, preliminary screening of the plants proposes that *Labisia pumila* var. *alata* (LPva) is more commonly used in traditional medicine preparations. Thus, a variety of research methodologies and data, such as quality control of raw materials based on pharmacognostical evaluation, extraction techniques and parameters, and tablet formulations, have been acquired for the purpose of proper standardization to ensure the quality, safety and efficacy of herbal products containing LPva (Jamal, 2006).

Excessive exposure to UV irradiation is considered to be the primary cause of the skin damage that gives rise to premature skin aging called photoaging. This skin illness is characterized by coarse wrinkles, roughness, laxity and also pigmentation (Choi *et al.*, 2010). Recent study has reported that *L. pumila* is consisted mainly of bioactive compounds such as phenolic acids, flavonoids and resorcinols (Ibrahim *et al.*, 2010). These compounds have been implicated as natural antioxidants, which can safely interact with free radicals and destroy their chain reactions before vital molecules could be damaged (Karimi *et al.*, 2011b). *L. pumila* extract also has been reported to be able to provide protection to human dermal fibroblast from UV-irradiated cell damage (Choi *et al.*, 2010). Thus, this study was carried out to investigate the anti-photoaging efficacy of LPva on UVB-irradiated human skin cells and to observe the morphological changes of the cells after being exposed to the UV radiation and after being treated with and without LPva plant extract.

Previous study has shown that several morphology studies have been made on photoaged skin cells. According to Scharffetter-Kochanek *et al.* (2000),
photoaged skin showed significant changes in the cellular component and connective tissue extracellular matrix, with an accumulation of disorganized elastin and severe loss of interstitial collagens. The fibroblast cells which are located in the dermis layer of the skin adopt a stellate phenotype and show a highly activated rough endoplasmic reticulum, indicating an increased biosynthetic activity (Uitto, 1986). The increments of mast cells and neutrophils have also been reported in photoaged skin (Kligman and Murphy, 1996). According to Danno and Horio (1982), several triggers such as UV irradiation are known to cause apoptosis in photoaged skin cells. The morphological features of the photoaged skin include the aggregation of nuclear chromatin and concentrated cell body when examined by electron microscopy (Olson and Everett, 1975). In some skin diseases such as basal cell carcinoma, squamous cell carcinoma and malignant melanoma, apoptosis-like changes are commonly observed, whose cells are called ‘dyskeratotic cells’ (Lever and Schaumburg-Lever, 1990). According to Baba et al. (1996), apoptotic cells are also observed morphologically even in non-irradiated cells. Thus, this suggests that slow apoptosis may occur consistently in the epidermis to regulate the cell population.

1.3 Problem Statement

The effect of ozone depletion resulting from nuclear explosions, the release of chlorofluoromethanes from refrigerants and aerosol sprays into the atmosphere has raised the concerns about the effects of increased exposure of human to ultraviolet (UV) irradiation. One anticipated effect of this phenomenon promotes some types of skin diseases in human such as premature skin aging or also known as photoaging. Recent studies have shown that some natural resources of medicinal plants such as L. pumila can repair UV-irradiated skin damage. However, the efficacy of this medicinal plant on biochemical and morphological changes of UVB-irradiated human skin cells after being treated with L. pumila extract has not yet been studied into detail.
Recent studies in skin biology have increased the understanding on skin homeostasis, aging process and also the mechanisms by which ultraviolet radiation contributes to photoaging and cutaneous disease. Thus, these have made the study of skin biology essential in order to develop a diversity of treatments that aim at preventing aging and rejuvenating the skin (Rabe et al., 2006). The search for skin rejuvenation is as old as humankind and is told in ancient stories, including the Greek Argonauts and the Fountain of Youth, where extensive efforts taken to restore youth are illustrated (Gonzales-Ulloa and Flores, 1965). Starting from this, individuals who are seeking treatment for reversal of age-associated changes in skin have become more and more. Thus, recent advances in skin biology have elucidated mechanisms by which photoaging occurs and given rise to new treatments to prevent and reverse this process (Rabe et al., 2006).

Up until now, the study of morphological and biochemical changes on human skin cells upon the treatment of local herbs such as *L. pumila* extract has yet to be studied into detail. Thus, this study was conducted in order to investigate the effect of *L. pumila* extract upon the morphology and biochemistry of the cells before and after being exposed to UVB irradiation. The morphology and biochemistry evaluations on the photoaged skin cells towards the treatment of *L. pumila* extract are essential in order to provide a strong scientific evidence of its efficacy on rejuvenating the skin cells and support the data that have been obtained by a number of researchers regarding this plant extract. Besides, in line with today high-end technology, it is better for humankind to manipulate the Mother Nature’s resource instead of using chemical substitutes which usually come with various side effects. In addition, this plant’s extract benefits should be explored due to the abundance of this plant in this country, and the advantages of this plant will never be known if it is not studied in details. Thus, this study is crucial in order to provide people with sturdy information of this plant extract on human skin vitality.
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