BACKHOUSIA CITRIODORA EXTRACT IN PROTECTING SKIN DAMAGE FROM PHOTOAGING

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DEDICATION

This thesis is dedicated to me, who are keep on fighting to complete the master journey. The quote of myself is when I feel like quitting, I will think about the reason why I started. I also dedicated this thesis for my son, Iffat Alfariz, who taught me that life is not easier.

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ABSTRACT

The aim of this study was to evaluate the potential of *Backhousia citriodora (B. citriodora)* extract in protecting skin damage from photoaging effect caused by ultraviolet B (UVB) irradiation. Photoaging is skin premature aging caused by repetitive exposure of sun which contains ultraviolet rays that can lead to photoaging effects. The presence of antioxidants can slow down the photoaging effects because the antioxidants can act as neutralizer for the skin. Many studies had been carried out on plants for development of natural antioxidants. In this study, *B. citriodora* extract was used as a source of natural antioxidants and the percentage yield obtained from ultrasonic-assisted extraction was 20.6 % with total extract 5 g. The extract was tested for the most abundant bioactive compound existed in the extract by using gas chromatography-mass spectrometry. The extract consisted of geranial (23.2 %), neral (19.7 %), 2-methyl-2-pentanal (10.1 %), geranic acid (1.9 %) and linalool oxide trans (0.02 %). The bioactive compounds found have good potential in cosmetics application and attribute to antioxidant activity. *B. citriodora* extract showed strong antioxidant activity with IC_{50} values 2.59 ± 0.532 µg/mL and 34.53 ± 1.14 µg/mL in 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid assays, respectively. The cytotoxicity of *B. citriodora* extract was evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in both human skin fibroblast and immortalized human keratinocytes cells. The results showed high percentage of cell viability using low concentrations of *B. citriodora* extract. The skin collagen increased from 40 % to 80 % when tested by using sircol soluble collagen assay. *B. citriodora* extract restored the synthesis of collagen initially reduced by UVB irradiation. Next, *B. citriodora* extract inhibited the human matrix metalloproteinase-1 (MMP-1) expression by 10%, indicated that *B. citriodora* extract inhibited the MMP-1 expression under the experimental condition used. In elastase assay, *B. citriodora* extract had higher elastase inhibition rate (>80 %) at 0.05 µg/mL compare to *Epigallocatechin gallate* (80 %). Therefore, it was concluded that *B. citriodora* extract can protect collagen and elastin from degradation, thus protect the skin from aging. The inflammatory cytokines were investigated using human tumour necrosis factor alpha (TNFα) and interleukin 6 (IL6). The TNFα and IL6 expression were reduced by 10 % and 70 %, respectively at concentration 7.81 µg/mL. The data demonstrated that *B. citriodora* extract can be used as an anti-inflammatory agent at low concentration. These results suggested that *B. citriodora* extract could be used as a potential antiaging and anti-inflammatory agent in cosmeceutical products.

ABSTRAK

Tujuan kajian ini adalah untuk menilai potensi ekstrak *Backhousia citriodora* (*B. citriodora*) dalam melindungi kerosakan kulit daripada kesan penuaan foto yang disebabkan oleh penyinaran ultraungu B. Penuaan foto adalah penuaan pramatang kulit yang disebabkan oleh pendedahan berulang terhadap sinar matahari yang mengandungi sinar ultraungu yang boleh menyebabkan kesan penuaan foto. Kehadiran antibahan pengoksidaan dapat melambatkan kesan penuaan foto kerana antibahan pengoksidaan dapat bertindak sebagai peneutralan untuk kulit. Banyak kajian telah dijalankan ke atas tumbuhan untuk menghasilkan antibahan pengoksidaan semulajadi. Dalam kajian ini, ekstrak *B. citriodora* digunakan sebagai sumber antibahan pengoksidaan semulajadi dan peratusan yang diperoleh dari pengekstrakan berbantu ultrasonic adalah 20.6 % dengan jumlah ekstrak 5 g. Ekstrak yang mengandungi sebatian bioaktif paling banyak diuji dengan menggunakan kromatografi gas-spektometri jisim. Sebatian bioaktif yang terdapat dalam ekstrak terdiri daripada geranial (23.2 %), neral (19.7 %), 2-metil-2-pentana (10.1 %), asid geranik (1.9 %) dan linalool oksida trans (0.02 %). Sebatian bioaktif tersebut mempunyai potensi yang baik dalam aplikasi kosmetik dan sifat untuk aktiviti pengoksidaan. Ekstrak *B. citriodora* menunjukkan aktiviti antibahan pengoksidaan yang kuat dengan nilai IC_{50} 2.59 ± 0.532 μg/mL dan 34.53 ± 1.14 μg/mL masing-masing dalam 2,2-difenil-1-pikrilhidrazil dan ujian 2,2'-azino-bis-(3-etilbenzotiazolin-6-sulfonik. Sitotoksisitas ekstrak *B. citriodora* dinilai dengan menggunakan ujian 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida ke atas kedua-dua fibroblast kulit manusia dan sel keratinosit manusia. Hasil kajian menunjukkan peratusan sel hidup yang tinggi dengan menggunakan kepekatan ekstrak *B. citriodora* yang rendah. Kolagen kulit meningkat daripada 40 % kepada 80 % apabila diuji menggunakan kolagen larut sirkol. Ekstrak *B. citriodora* mengembalikan sintesis kolagen yang pada mulanya berkurangan disebabkan sinaran ultraungu B. Seterusnya, ekstrak *B. citriodora* menghalang ekspresi matrik metalloproteinase-1 (MMP-1) manusia sebanyak 10 %, menunjukkan bahawa ekstrak *B. citriodora* menghalang ekspresi MMP-1 di bawah keadaan eksperimen yang digunakan. Dalam ujian elastase, ekstrak *B. citriodora* mempunyai kadar perencatan elastase yang lebih tinggi (> 80 %) pada 0.05 μg/mL berbanding *Epigallocatechin gallate* (80 %). Oleh itu, disimpulkan bahawa ekstrak *B. citriodora* dapat melindungi kolagen dan elastin daripada degradasi, seterusnya melindungi kulit dari penuaan. Sitokin keradangan dikaji menggunakan faktor tumor nekrosis alpha (TNFα) dan interleukin 6 (IL6). Ekspresi TNFα dan IL6 masing-masing berkurangan sebanyak 10 % dan 70 %, pada kepekatan 7.81 μg/mL. Data menunjukkan bahawa ekstrak *B. citriodora* boleh digunakan sebagai agen anti-radang pada kepekatan yang lebih rendah. Hasil kajian ini menunjukkan bahawa ekstrak *B. citriodora* dapat digunakan sebagai agen antipenuaan dan anti-keradangan yang berpotensi dalam produk kosmetik.

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LIST OF ABBREVIATIONS

UV	-	Ultra-Violet
UVA	-	Ultra-Violet (A)
UVB		Ultra-Violet (B)
GCMS	-	Gas Chromatography-Mass Spectrometry
DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
ABTS	-	2,2-Azino-Bis-3-Ethylbenzothiazoline-6-sulphonic Acid
MTT	-	Thiazolyl Blue Tetrazolium Blue
HSF	-	Human Skin Fibroblast
Hacat	-	Immortalized Human Keratinocytes
MMP	-	Matrix Metalloproteinase
EGCG	-	Epigallocatechin Gallate
TNFα	-	Tumour Necrosis Factor Alpha
IL6	-	Interleukin 6
UAE	-	Ultrasonic-Assisted Extraction
ICH	-	International Conference on Harmonization
DHA	-	Dynamic Headspace Analysis
ECM	-	Extracellular Matrix
ROS	-	Reactive Oxygen Species
AP	-	Activator Protein
TGF	-	Transforming Growth Factor
ELISA	-	Enzyme-Linked Immunosorbent Assay
DMSO	-	Dimethyl Sulfoxide
DMEM	-	Dulbecco's Modified Eagle Medium
FBS	-	Fetal Bovine Serum
PBS	-	Phosphate-Buffered Saline
ATCC	-	American Type Culture Collection

LIST OF SYMBOLS

℃	-	Degree Celsius
α	-	alpha
L	-	Litre
ml	-	millilitre
mg	-	milligram
µg	-	microgram

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The global skin care business activity represented a largely untapped market opportunity for natural, plant derived extracts with antiaging and antioxidants bioactivity. Many skin care industries need almost pharmaceutical standards in terms of proof of safety and efficacy as potential sources of antiaging agents which can be used in protecting skin damage from photoaging caused by Ultraviolet (UV) irradiation.

Photoaging is skin premature aging caused by repetitive sun exposure to UV radiation, such as Ultraviolet A (UVA) and Ultraviolet B (UVB) rays. Besides, the skin aging is also caused from sources of artificial UV. The word "photo" is obtained from the Greek word "phos" which means "light" (American Academy of Dermatology, 2020). Therefore, the skin aging is affected by light. Photoaging is different from natural or chronological aging, as the damaging effects of UV rays from the sun. Besides, exposure to UV rays, whether UVA or UVB, from sunlight accounts for 90 percent of the symptoms of premature skin aging including dark spots, wrinkles, leathery and droopy skin, and others (Grabel, 2019). Although UVA rays is more harmful to skin, UVB rays are known to stimulate Tumour Necrosis Factor alpha (TNFα), Interleukin 1 (IL-1) and Interleukin 6 (IL-6), thus increasing the matrix metalloproteinase 1 (MMP-1) that can cause skin aging. The intensity of UVB rays vary by season, location and time and it can cause permanent damage to skin that can lead to photoaging effects. Photoaging become visible on body parts that people can see, such as the neck, back of the hands and face. Addition, the lips also can show the excessive sun damage signs. These exposed areas usually received a lot of sun exposure.

Antioxidants play an important role in neutralizing free radical species which are produced as end or by-products of normal biochemical reactions in normal system (Jimenez-Estrada *et al.*, 2013). High amounts of free radical molecules cause oxidative stress in cells which result in damaging essential macromolecules including DNA, lipids, and proteins. The damage of macromolecules leads to antiaging, inflammation and many degenerative conditions such as Parkinson's diseases, atherosclerosis, aging, immunosuppression, ischemic heart disease, diabetes, hair loss, membrane lipid peroxidation, and decreased membrane fluidity (Patel *et al.*, 2010). Reactive oxygen species are also reported as carcinogenic and mutagenic agents [Aguirre and Borneo, 2010).

Backhousia citriodora (B. citriodora) (lemon myrtle) is an Australian plant, native to subtropical areas of eastern Australia. In 2009, the plant was introduced to Malaysia by Qzen (M) Sdn. Bhd makes it one of the potential crops in the country. The leaves contain the highest amount of citral (>90 %) compare to any plant known in the world, leading to a general description that it is 'lemony than lemon'. The leaves of this plant are widely used as a bush food and as a component of toiletries and cosmetics products like cleanser, moisturizer, hand wash and hair products. *B. citriodora* can be smashed into almost any form, wet or dry. Previous study by Cock, IE. (2008) have demonstrated the antibacterial activity of *B. citriodora* leaves.

Recently, many kinds of biomolecules have potential in antioxidant activity are being applied in anti-aging study. But there is no specific group of chemical constituents of antioxidant in *B. citriodora* extract that have been found to prevent the photoaging effects. The *B. citriodora* extract is subjected to GC-MS analysis to explore the bioactive compounds exist in the essential oils that contribute to antioxidant activity. *B. citriodora* have been reported to have high antioxidant response which was concluded to have cinnamic acids and flavonoids (Konczak *et al.*, 2010). Besides, the dominant compound of *B. citriodora* hydrophilic extract is neral (42.5 %), geranial (48.5 %) (Southwell, Russel, and Smith, 2003). Other percentages of compound found in the extract was minor constituents (0.1-2.3 %). These phytochemicals are reported to possess numerous health- enhancing properties. For example, ellagic acid is a potent

antioxidant, exhibits anti-inflammatory, antimicrobial, and prebiotic effects (Corbett *et al.*, 2010; Landete, 2011; Lee *at al.*, 2010; Rosillo *et al.*, 2012). Interestingly, no reports of antiaging use of *B. citriodora* were found in the literature, although the leaves were used in cosmetics application. Therefore, this study was focused on evaluating the potential of *B. citriodora* extract to repair UVB-irradiated skin damage.

The potential of *B. citriodora* extract to possess antiaging and inflammatory activity, several biochemical assays were studied to detect, quantify, and analyze the activities. For example, Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive biochemical assay that uses enzyme-conjugated antibodies, with antibodies bound to a solid support. During the ELISA analysis, a controlled sequence of biochemical reactions generates a signal that can be easily quantified and interpreted the amount of analyte in the sample. This technique is commonly used to detect the activity of substances, like antibodies, proteins, hormones, and peptides by measuring their signals (Felgueiras *et al.*, 2018). In this study, the antiaging and inflammatory activity were mostly used the ELISA technique to identify the required substances. Other than that, cytotoxicity activity is also important to assess the potential risk of tested extract on the skin. Mostly, in cosmeceutical application, the safety of the product to be applied on the skin become priority. Therefore, the suitable assay was chosen to evaluate the *B. citriodora* extract on cytotoxicity, antiaging and inflammatory activity.

1.2 Problem Statement

Malaysian's herbal market is fill with a lot of local herbs, mainly in nutraceutical, pharmaceutical and cosmeceutical fields. Many herbal products claimed to have lots of biological advantages, for example supplement high in antioxidants. The increasing awareness on consuming natural products leads to high demand on natural and herbal market. In the cosmetics industry, hundreds of substances are used to impose various effects on the human skin. These substances range from purely natural compositions extracted from nature in their original state to purely synthetic compositions which have been produced from synthetics through a complex series of chemical reactions.

Cosmeceutical products have a direct physical relationship with human, as they use the products daily on their skin and bodies. The chemical in cosmetics products did not just lay on the skin surface without penetrating the skin. If people consider the substances being internalized by the body, absorbing mineral pigments or essential oils, plant oils and waxes are better than absorbing synthetic chemicals and petroleum by-products. In many cases, duration effects of many chemical additives in cosmeceutical application are not known. But other chemical additives are known to cause cancer in human such as carcinogens. Chemical-free products, natural foods and exercise helped the skin and body into source of beauty and healthy (Fairley and Josephine, 2001).

Skin produces free radicals or reactive oxygen species due to repeated sun exposure, which leads to oxidative stresses and inflammatory responses in the dermal or epidermal layer of the connective tissues resulting aging and damage to cell membranes, lipids, proteins, and DNA (Yamamoto, 2001). In human skin fibroblast (HSF) and immortalized human keratinocytes (Hacat), reactive oxygen species (ROS) were stimulated by UV irradiation, which lead to signal transduction and the up-regulation of the AP-1 transcription factor (Hasham *et al.*, 2013).

According to Oh *et al.* (2010), AP-1 controls the transcription of matrix metalloproteinase (MMPs). UVB rays are known to stimulate the MMPs which can cause degradation of collagen. Therefore, there is a need to find new skin-care cosmetic ingredients from natural resources that can prevent photo-induced biological damages and inhibit the enzymatic factors in the process of photoaging.

B. citriodora extract can be used as antiaging treatment because the extract consists of bioactive compound responsible for antioxidant activity such as linalool oxide and citral. *B. citriodora* also has significant antimicrobial activity that has potential as a surface disinfectant, antiseptic or for inclusion in foods as a natural antimicrobial agent (Wilkinson *et al.*, 2003). The results of this study indicated that *B. citriodora* extract possesses remarkable antioxidant, and antimicrobial properties and serve as the viable source for the skin aging protection agents.

1.3 Objective of Study

The objective of this study is to evaluate the potential of *Backhousia citriodora* extract in protecting skin damage from photoaging caused by UVB irradiation.

1.4 Scope of Study

The scopes of this research area are:

1. Preparation of *B. citriodora* leaves, chemical reagents, and consumables materials.

2. Extraction of *B. citriodora* leaves by using Ultrasonic-Assisted Extraction (UAE) with methanol as solvents, solid to solvent ratio 14:1, and time taken is 54.87 minutes at 60 ℃.

3. Analysis of bioactive compounds in *B. citriodora* extract by using Gas chromatography- mass spectrometry (GCMS).

4. Determination of antioxidant activity of *B. citriodora* extract by using the DPPH and ABTS radical scavenging activity.

5. Evaluation of cytotoxicity activity of *B. citriodora* extract by using MTT assay on human skin fibroblast (HSF) and immortalized human keratinocytes (Hacat) at concentration of 1000 µg/mL –31.25 µg/mL.

6. Investigation of antiaging activity of *B. citriodora* extract on human skin fibroblast (HSF) and immortalized human keratinocytes (Hacat) by using Sircol soluble collagen assay, human MMP-1 ELISA, and elastase assay.

7. Investigation the inflammatory effect of *B. citriodora* extract by using Human Tumour Necrosis Factor alpha (TNF-α) and Human Interleukin 6 (IL6) ELISA kit.

1.5 Significant of Study

This research will be a significant endeavour in promoting *B. citriodora* extract as an antiaging and inflammatory agent that can protect the skin damage caused by UVB irradiation. The scientific evidences found on HSF and Hacat in the study can be used to commercialize the extract in cosmeceutical and nutraceutical application. Other than that, this study also beneficial to research and development (R&D) industry as a reference by providing important information regarding the *B. citriodora* extract, antiaging and inflammatory activity.

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