

CYTOXICITY AND INHIBITORY EFFECTS OF *BACKHOUSIA CITRIODORA*
EXTRACT ON MELANIN BIO-SYNTHESIS OF B16F1 MELANOMA CELL

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ABSTRACT

Tyrosinase inhibitors are gaining attention due to their important role in inhibiting melanin production. Tyrosinase inhibitors are widely used in the cosmetic industries. However, some of the well-known inhibitors are reported to be harmful and can cause undesirable side effects. Thus, application of natural ingredients in formulation has garnered massive attention among consumers to promote health and safe lifestyle. *Backhousia citriodora* was first introduced in Malaysia by Qzen (M) Sdn. Bhd. in 2009, in the form of stem cuttings. In this study, the extract of *B. citriodora* was evaluated for its biological activities. 2,2-diphenyl-1-picrylhydrazyl assay of the extract exhibited good potential for further development as antioxidant agent with IC₅₀ values of 53.94 mg/ml, compared to ascorbic acid (83.98 mg/ml) and trolox (77.59 mg/ml). In addition, the inhibitory effect of *B. citriodora* extract on melanogenesis was evaluated using B16F1 melanoma cell. The cytotoxicity study was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, water soluble tetrazolium salt assay and Sulforhodamine B assay. The cell viabilities treated with *B. citriodora* extract with concentration at 0.03125 - 0.10 % (v/v) showed no significant effect to the cell compared to the non-treated cell. This indicates that *B. citriodora* extract is non-cytotoxic to the cells under the experimental condition used. Melanogenesis measured in α -MSH-induced B16F1 melanoma cells revealed that by *B. citriodora* extract treatment at 0.0625 - 0.10 % (v/v), the secreted melanin and intracellular melanin were significantly reduced in dose dependent manner. It can be also deduced that the inhibitory effect of *B. citriodora* extract is stronger than kojic acid and arbutin (positive control used). Furthermore, cellular tyrosinase activity exhibited a similar profile to melanin content. These results showed that *B. citriodora* extract has the ability to suppress cellular melanin synthesis in B16F1 cells.

ABSTRAK

Perencat tirosinase semakin mendapat perhatian disebabkan oleh peranan pentingnya dalam merencatkan pembentukan melanin. Perencat tirosinase digunakan secara meluas dalam industri kosmetik. Walau bagaimanapun, beberapa perencat yang terkenal dilaporkan berbahaya dan boleh menyebabkan kesan sampingan yang tidak diingini. Oleh itu, penggunaan bahan semulajadi dalam perumusan kosmetik telah mendapat perhatian dikalangan pengguna bagi menggalakkan kehidupan dan gaya hidup yang selamat. *Backhousia citriodora* mula diperkenalkan ke Malaysia oleh Qzen (M) Sdn. Bhd. pada tahun 2009, dalam bentuk keratan batang. Dalam kajian ini, aktiviti biologi ekstrak *B. citriodora* telah dikaji. Ujian 2,2-difenil-1-pikrilhidrazil menunjukkan bahawa ekstrak tersebut mempunyai potensi yang baik sebagai agen antioksidan dengan nilai IC_{50} 53.94 mg/ml, berbanding asid askorbik (83.98 mg/ml) dan trolox (77.59 mg/ml). Di samping itu, kesan perencatan ekstrak *B. citriodora* terhadap melanogenesis telah dinilai menggunakan sel melanoma B16F1. Kajian sitotoksik dilakukan menggunakan kaedah 3-(4,5-dimetilthiazol-2-yl)-2,5-difeniltetrazolium bromida, garam larut air tetrazolium dan ujian Sulforhodamine B. Kebolehhidupan sel yang dirawat dengan ekstrak *B. citriodora* pada kepekatan 0.03125 - 0.10 % (v/v) tidak menunjukkan kesan yang menjejaskan sel berbanding dengan sel yang tidak dirawat. Ini menunjukkan bahawa ekstrak *B. citriodora* tidak sitotoksik kepada sel-sel dalam keadaan eksperimen yang digunakan. Melanogenesis diukur dalam sel-sel melanoma B16F1 yang diinduksi oleh α -MSH mendedahkan bahawa melalui rawatan ekstrak *B. citriodora*, pada kepekatan 0.0625 - 0.10 % (v/v), rembesan melanin dan melanin intraselular berkurangan bergantung kepada dos. Ia juga boleh disimpulkan bahawa kesan perencatan ekstrak *B. citriodora* adalah lebih kuat berbanding asid kojik dan arbutin (kawalan positif yang digunakan). Selanjutnya, aktiviti tirosinase selular menunjukkan profil yang sama dengan kandungan melanin. Keputusan ini menunjukkan bahawa ekstrak *B. citriodora* mempunyai keupayaan untuk menyekat sintesis melanin selular dalam sel B16F1.

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LIST OF ABBREVIATIONS

ACTH	-	Adrenocorticotrophic hormone
ATCC	-	American Type Culture Collection
cAMP	-	cyclic AMP
CRE	-	cAMP response element
DCT	-	Dopachrome Tautomerase
DHI	-	5,6-dihydroxyindole
DHICA	-	5,6-dihydroxyindole-2-carboxylic acid
DMEM	-	Dulbecco's Modified Eagle's Medium
DMSO	-	Dimethyl sulfoxide
DPPH	-	2,2-Diphenyl-1-Picrylhydrazyl
FBS	-	Fetal Bovine Serum
FDA	-	Food and Drug Administration
GCMS	-	Gas Chromatography Mass Spectrometer
ICH	-	International Conference Harmonization
L-DOPA	-	L-3,4-dihydroxyphenylalanine
MC1R	-	Melanocortin 1 receptor
MITF	-	Microphthalmia-Associated Transcription Factor
MOH	-	Ministry of Health
MTT	-	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NPCB	-	National Pharmaceutical Control Bureau
P	-	Phosphorylation
PBS	-	Potassium Buffered Saline
PKA	-	Protein Kinase A
ROS	-	Reactive Oxygen Species
SD	-	Standard Deviation
SEM	-	Standard Error of The Mean
SRB	-	Sulforhodamine B
TYR	-	Tyrosinase
TYRP1	-	Tyrosinase-Related Protein 1
WST-1	-	Water Soluble Tetrazolium Salts

UAE	-	Ultrasonic-Assisted Extraction
α -MSH-	-	alpha-Melanocyte Stimulating Hormone-

LIST OF SYMBOLS

α	-	Alpha
$^{\circ}\text{C}$	-	Degree celcius
$\mu\text{g/ml}$	-	Microgram per mililitre
μL	-	Microlitre
%	-	Percent
R_1	-	Retention index
v/v	-	Volume per volume

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Cosmeceutical term is derived from the words cosmetic and pharmaceutical. It was introduced by dermatologist Dr Albert Kligman around the year 1984. Cosmeceutical products are marketed as cosmetics is said to contain biologically active ingredients like anti-aging and whitening creams which have drug-like or medicinal benefits. The term cosmeceuticals has round up to cover the functional cosmetic products that go above and beyond their intended function by offering additional therapeutic benefits (Park, 2009).

Natural personal care market is categorized into cosmetics, oral care, skin care, and hair care among others. As reported by grand view research in 2018, the skin care segment led the global market (Figure 1.1). From the segment, it shows that skin care is the most popular among other personal care in terms of market size and share thus make it an important factor to produce high quality product which related to skin care basis.

Therefore, it can be said that the rising skin concerns is a major driving force which influences consumer preference and demand positively. Furthermore, rising demand for organic, herbal, and natural beauty products has created growth opportunities for researchers to innovate and develop new products in accordance with consumer preferences. Besides developing high quality product for consumers, it is also crucial for researchers to ensure that the source of the active compound is safe as well.

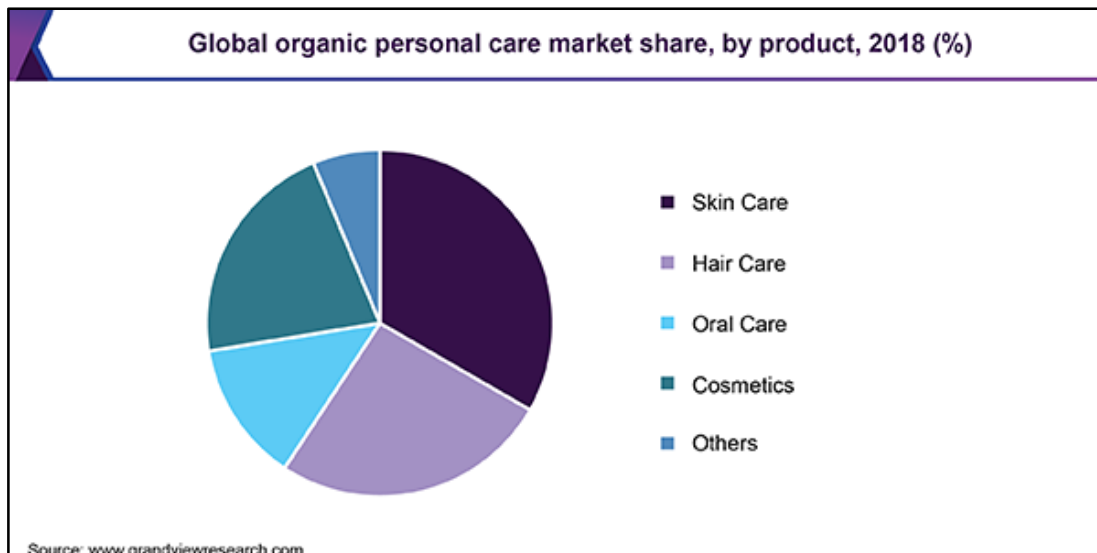


Figure 1.1 Organic personal care market size, share: industry report, 2019-2025. (<https://www.grandviewresearch.com/>)

Having a fair and radiant skin is a trend in Malaysia nowadays. Most of the local market products claim to be effective in lightening the skin. Consumers are also tend to choose the natural based products rather than toxic and harmful chemicals. Great sources of antioxidant from plant and animal derived products rich in compounds that can improve complexion. Other than that, it also moisturize the skin, reduce wrinkles and fine lines, and thus gives elasticity effects to the skin (Ferris, 2007).

The beauty industry is shaped by the ever changing customer demands. In recent years, the need of whitening effect in skin care product has risen to the top. Obsession towards fair and radiant looks on the skin especially for people in Asian region makes the demand of producing whitening product growing. One of the consumers concern on their skin is the uneven skin tone cause by pigmentation disorder.

Pigmentation disorder is defined as disturbances in human skin colour either increasing or reducing in melanin. It is a condition where the skin colour may appear darker or lighter than normal. In general, people with darker skin produce higher amount of melanin whereas people with pale skin produce very small melanin. Types of pigmentation include hyperpigmentation, hypopigmentation and depigmentation.

Disorders of pigmentation can result from migration abnormalities of melanocytes from the neural crest to the skin during embryogenesis. In addition, impairment of melanosome transfer to the surrounding keratinocytes, an alteration in melanin synthesis and a defective degradation or removal of melanin may lead to abnormal skin pigmentation. Immunologic or toxic mediated destructions of melanocytes can end in pigmentation disorders (Fistarol and Itin, 2009).

Skin pigmentation involve *de novo* synthesis of melanin in melanocytes and transfer of the synthesized melanin packed in melanosome to neighbouring keratinocytes and eventually turns the skin colour into a darker tone (Seiberg et al., 2000 and Hearing, 2005). There are three types of enzyme contain in melanosome that is tyrosinase, TRP1 and TRP2 (Kobayashi and Hearing, 2007).

Tyrosinase is a rate limiting enzyme involved in melanin bio-synthesis. It hydroxylates tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidized L-DOPA to DOPA quinone (Kim et al., 2006). Excessive accumulation of DOPA quinone generated from hydroxylation while oxidation of tyrosine forms DOPA chrome which conditionally exhausted cysteine and resulted in accumulation of brownish and black pigment called eumelanin. Another type of melanin called pheomelanin is produced through formation of 3 or 5 cysteinyl DOPA on condition of being existence of cysteine (Yamaguchi et al., 2007 and Miyamura et al., 2007). Hence, our skin colour can be determined by the ratio of both eumelanin and pheomelanin.

Melanin bio-synthesis can be inhibited by avoiding ultraviolet exposure, by inhibition of melanocyte metabolism and proliferation (Kim and Uyama, 2005), by inhibition of tyrosinase or by removal of melanin by corneal ablation. One of the factors to avoid melanin production is the inhibition of tyrosine oxidation catalysed by tyrosinase (Slominski et al., 2004).

Hyperpigmentation is the excessive pigmentation of the skin due to increase in melanin content in the body. Common causes of hyperpigmentation are overexposure to the sunlight, inherit, hormonal changes, picking at the skin, certain medications for

example antibiotics, hormone treatments, and anti-seizure drugs, inflammation and also skin wound such as acne vulgaris. However, there are options to overcome this condition which is by lightening the appearance of it.

Cosmetic treatment to even out the colour of the skin and diminish the prominence of skin discolorations is called skin bleaching. To change their appearance, some people apply skin lightener to their whole body, yet this can be unsafe. One of popular skin lighteners' active ingredient is mercury. A toxic and harmful agent like mercury can bring serious issues like psychiatric as well as neurological issues. Thus, skin discolouration can lead to mercury harming.

Hyperpigmentation can also be treated with certain prescription cream such as hydroquinone. It is said to be the most effective melanogenesis inhibitor in vitro and in vivo and is known to be a hazardous substance which has a carcinogenic effect on human skin. Hydroquinone used over the short term provides some skin lightening, but continued use can also lead to skin pigmentation. It also has other harmful effects such as skin sensitivity to light, kidney damage and severe allergic reaction.

Ministry of Health (MOH) with the cooperation of the National Pharmaceutical Control Bureau (NPCB), has listed beauty products in Malaysia which contain banned substances and harmful to consumers. These products especially skin lightening products that contain mercury and hydroquinone is among the products that are banned by the MOH. These harmful products are no longer allowed to be imported, manufactured, distributed and sold in Malaysia. Consumers are advised not to purchase and use the cosmetics products because it can cause serious adverse effects. Also, in the United States, the use of mercury in the product formulations for skin lighteners is illegal. However, in any case, some products produced outside the United States might even now contain mercury. This phenomenon has urged researchers to look for another potential tyrosinase inhibitor for cosmetic used.

Antioxidant is a molecule that inhibits the oxidation of other molecules. *Backhousia citriodora* (lemon myrtle) is claimed to have high antioxidant properties and has the highest ability to scavenge the oxygen free radicals (Konczak et al., 2010).

The formation of reactive oxygen species (ROS) is stimulated upon UV irradiation and known to be an early event caused by UV in skin. Enhanced amount of ROS probably triggers an inflammatory signaling pathway, which eventually causes downstream cellular activities leading to inflammation, skin aging, and melanogenesis. Therefore, ROS scavenger become a primary target to prevent photoaging and melanin synthesis (Oh et al, 2011).

Backhousia citriodora (lemon myrtle) widely found in Queensland, Australia regions. In 2009, the plant was introduced to Malaysia by Qzen (M) Sdn. Bhd. The success in breeding makes it one of the potential crops in the country. The introduction of the newly breed plant in Malaysia which is the lemon myrtle itself make it useful to study the benefits of the active compound present in the plant. *Backhousia citriodora* (lemon myrtle) extract may be used in order to inhibit melanin production. Thus, in this study, *Backhousia citriodora* extract was investigated whether it could inhibit melanin bio-synthesis in human skin.

1.2 Problem Statement

There are a lot of choice of cosmetic products on the shelf nowadays. Most of them are made of chemical base ingredients. Chemical based ingredients might cause long term effects or have side effects for the user in the long term time. Usage of harmful chemical products might cause toxic to the skin. This toxic condition will leads to skin related problem to consumers.

Therefore, there is the need to find a safer choice of cosmetic products with natural based ingredients. Natural ingredients is said to provide a better choice to human and less harmful to the skin as well. Natural based products are more safe and suitable to be used as the substitute of the chemical based products. Hence, it is crucial for researchers to overcome this problem by looking for an alternative ingredients from natural resources which is safer in order to cater the problem.

There are several diseases related to pigmentation disorder like freckles, melasma, vitiligo and albinism. For example, melasma (a term derived from the Greek word melas, meaning black) is a common acquired hypermelanosis that occurs exclusively on sun-exposed areas, mostly on the face and occasionally on the neck and forearms. The exact cause of melasma remains elusive, but the two most important factors implicated in its etiopathogenesis are sunlight and genetic predisposition (Electra et al., 2014).

In a way to find suitable phytochemical compounds that modulate melanogenesis activity, there are some characteristics need to be focused. One of the factors that contribute to melanin production is the exposure of skin to ultraviolet. Long exposure to sunlight may lead to harmful skin cancer. It will cause adverse effects by oxidative stress such as suppression of the skin immune system and DNA damage. Free radicals up-regulating the m-RNA level for tyrosinase which is a rate limiting enzyme in melanin bio-synthesis (Costin and Hearing, 2007). There are several problem related to chronic skin disease. For example skin cancer, melasma and vitiligo. Therefore it is crucial to screen wether *Backhousia citriodora* can be used as one of the cosmetic ingredients in skin lightening product.

Backhousia citriodora (lemon myrtle) oil has been shown to possess antimicrobial activity and consequently be an effective topical antimicrobial agent (Hayes and Markovic, 2002). Antifungal activity also has been reported by Jenny (2003). The essential oil of *Backhousia citriodora* demonstrated activity against six of the organisms (*A. faecalis*, *E. coli*, *E. coli type B*, *S. typhimurium*, *S. sonnei*, *A. niger*, and *T. tonsurans*) at a concentration of 1 %. In addition, *Backhousia citriodora* (lemon myrtle) essential oils are also used extensively in pharmacy, medicine, food, beverages, cosmetics, perfumery and aromatherapy. It has been proven that the essential oil of *Backhousia citriodora* (lemon myrtle) is effective in the treatment of molluscum contagiosum in children (Burke et al., 2004).

Backhousia citriodora could treat molluscum contagiosum, strong candidate as antimicrobial agent. However, the potential of plant to inhibit melanin bio-synthesis has yet to be reported. For *Backhousia citriodora* (lemon myrtle) extract to be used as

an effective topical application, a toxicity safety assessment is required. Thus, the analysis of cytotoxicity of the extract should be carried out. Studying cytotoxic effects means determining harmful effects on cells in vitro. The aim is to prove the safety of pharmaceutical or cosmeceutical products or food stuff, to screen for new biological active compounds as well as to prove the biocompatibility of medical materials.

Melanogenesis is a complex biosynthesis process where the pigment melanin is produced in melanosome by melanocyte cell. It is likewise can be said as the whole process leading to the development of dark macromolecular colours. Melanin can be found in skin, hair and eyes as the primary determinant of the colours. Melanin mainly plays a photo-protective role due to its ability to absorb the radiation of ultraviolet. The accumulation of unusual amounts of melanin in various parts of the skin might become an esthetical problem. Melanin overproduction stimulated by scarring, hormones or excessive exposure to sunlight which will result in pigmented patches of skin. Therefore, a few studies have been focusing on the inhibition of tyrosinase activity and the prevention of abnormal pigmentation.

Pattern towards natural skin lightening solution is growing as more individuals look for other approaches to treat issues connected with uneven skin tone, dark skin pigmentation, skin inflammation scars and prolonged sun damage without the utilization of hazardous chemicals. Therefore in this study, the inhibition effect of *Backhousia citriodora* (lemon myrtle) extract on melanin bio-synthesis and tyrosinase activity of B16F1 melanoma cells investigated. These findings will lead to a new solution to substitute the hazardous and toxic chemical in skin lightening products.

1.3 Objective of Study

The objectives of this study are:

1. To determine the cytotoxicity effect of *Backhousia citriodora* (lemon myrtle) extract on B16F1 melanoma cells.
2. To investigate the inhibitory effect of *Backhousia citriodora* (lemon myrtle) extract on melanin bio-synthesis and tyrosinase activity of B16F1 melanoma cells.

1.4 Scope of Study

The scopes of the study are:

1. Extraction of *Backhousia citriodora* (lemon myrtle) leaves by using ultrasonic assisted extraction with type of solvent used is methanol, solvent to solid ratio is 14:1 and extraction time is 54.87 minutes at 60 °C.
2. Evaluation of cytotoxicity effects of *Backhousia citriodora* (lemon myrtle) extract on B16F1 melanoma cells by using MTT assay, WST-1 assay and SRB assay on the cell viability at concentration 0.03125 – 0.100 % v/v.
3. Evaluation of antioxidant activity of *Backhousia citriodora* (lemon myrtle) extract carried out by DPPH radical scavenging activity.

4. Evaluation of melanin bio-synthesis activity of *Backhousia citriodora* (lemon myrtle) extract on B16F1 melanoma cells is carried out by the measurement of secreted melanin content and the intracellular melanin assay for the formation of melanin in the cell. Evaluation of mushroom tyrosinase assay and intracellular tyrosinase activity assay which acted as the enzymatic assay for the tyrosinase inhibition activity.

1.5 Significance of Study

This research is important to give new findings on the effects of *Backhousia citriodora* (lemon myrtle) extract on melanin bio-synthesis of B16F1 melanoma cell culture. The outcome of the study provide the scientific evidence on the inhibitory effects of the extract to the cells which will benefit for the future cosmetic industries. Also, the findings of the cytotoxicity effects of the *Backhousia citriodora* (lemon myrtle) extract benefits the industry by providing the most suitable concentration of the extract that could potentially be used in the cosmetics formulation.

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