UNIVERSITI PUTRA MALAYSIA

INVESTIGATION OF MELANOGENESIS USING NEONATAL NORMAL HUMAN EPIDERMAL MELANOCYTE TREATED WITH MAWA YOUNG COCONUT WATER

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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Chair : Professor Fauziah Othman, PhD
Faculty : Medicine and Health Siences

The actual color of skin is determined by the type, size and amount of melanin synthesized by melanocytes, and also deposition pattern of melanin in the surrounding keratinocytes. Melanin is synthesized through a series of oxidative reactions and by the enzyme called tyrosinase. Besides turosinase, other melanogenic enzymes including tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) also known as dopachrome tatumerase (DCT) are involved in melanin synthesis pathway. Overproduction and aggregation of melanin in the human skin, can cause dark-skinned and also aesthetics problems which encourage researches to develop cosmetic agents with high efficacy and less side effects. Hence, inhibition of tyrosinase activity or melanogenic pathways to have skin lightening are challenging subjects challenge for many researchers. Today, many famous whitening agents such as kojic acid and hydroquinone have been used commercially in whitening creams and other products such as lotion. Due to some adverse effects of theses whitening agents such as poor penetration and skin irritation, a natural tyrosinase inhibitor with less harmful side effects, and also low cost producer are always in demand. The objective of this study was to investigate the effect of young coconut water on melanogenesis using neonatal normal human epidermal melanocytes. In this study, cell viability assay was performed to investigate a safe concentration of young coconut water on neonatal normal human epidermal melanocyte. In addition, young coconut water evaluated for *in vitro* cellular tyrosinase activity and melanin content in neonatal normal human epidermal melanocyte. In the present study, the protein levels of tyrosinase and other tyrosinase enzymes including tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) also called dopachrome tatumearse (DCT) which are involved in melanogenesis pathway were determined using western blot method. In this study, skin melanocytes were treated with different concentration of young coconut water and compared with untreated cells. The result from MTT assay showed that young coconut water exhibited no cytotoxicity on melanocytes at 10 mg/ml and half-maximal cytotoxicity concentration (CC_{50}) was 13.12 mg/ml. This study indicated that young coconut water reduced the tyrosinase activity by inhibition of its activity with an IC_{50} (half-maximal inhibitory concentration) value of 10 mg/ml and also down regulated the protein level of tyrosinase. Results from western blot demonstrated that the protein level of tyrosinase related protein-2 significantly decreased at 8 and 10 mg/ml of young

coconut water by 0.351 and 0.280 fold, respectively. There was no significant reduction in protein level of tyrosinase related protein-1 (TRP-1). Although, young coconut water at low concentrations did not significantly reduce tyrosinase activity. In conclusion, young coconut water at 8 and 10 mg/ml obviously reduced the protein level of tyrosinase and tyrosinase related protein-2 (TRP-2) with more that 80% viability. This result indicated that young coconut water might be considered as a potential whitening agent in cosmetics.

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

KAJIAN TENTANG MELANOGENESIS MENGGUNAKAN MELANOSIT EPIDERMIS MANUSIA NORMAL NEONATAL DIRAWAT DENGAN AIR KELAPA MUDA MALAYSIA

Oleh

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Warna kulit asal ditentukan oleh jenis, saiz dan jumlah melanin disintesis oleh melanosit, dan juga pemendapan corak melanin dalam sekitar keratinosit. Melanin disintesis melalui siri tindak balas pengoksidaan dan oleh enzim dipanggil tirosinase. Selain tirosinase, enzim melanogenik lain termasuk protein tirosinase-1 (TRP-1) dan protein tirosinase-2 (TRP-2) juga dikenali sebagai dopachrome tatumerase (DCT) terlibat dalam laluan sintesis melanin. Pengeluaran berlebihan dan pengagregatan melanin dalam kulit manusia, boleh menyebabkan kulit hitam dan juga masalah estetika yang menggalakkan penyelidik untuk menghasilkan ejen kosmetik dengan keberkesanan yang tinggi dan kesan sampingan yang kurang. Oleh itu, perencatan aktiviti tirosinase atau laluan melanogenik untuk mempunyai kulit yang lebih cerah merupakan satu cabaran bagi ramai penyelidik. Kini, banyak agen pemutih terkenal seperti asid kojik dan hidrokuinon telah digunakan secara komersial dalam krim pemutih dan produk-produk lain seperti losyen. Berikutan beberapa kesan buruk agen pemutihan tesis seperti kurang penembusan dan kerengsaan kulit, perencat tirosinase semula jadi dengan kesan sampingan yang kurang berbahaya, dan juga pengeluar kos rendah adalah sentiasa dalam permintaan. Objektif kajian ini adalah untuk mengkaji kesan air kelapa muda terhadap melanogenesis menggunakan melanosit epidermis manusia normal neonatal. Dalam kajian ini, asai kebolehhidupan sel telah dijalankan untuk mengkaji kepekatan air kelapa muda yang selamat pada melanosit epidermis manusia normal neonatal. Selain itu, air kelapa muda dinilai untuk aktiviti sel tirosinase *in vitro* dan kandungan melanin dalam melanosit epidermis manusia normal neonatal. Dalam kajian ini, tahap protein tirosinase dan enzim tirosinase lain termasuk protein tirosinase-1 (TRP-1) dan protein tirosinase-2 (TRP-2) juga dipanggil dopachrome tatumerase (DCT) yang terlibat dalam laluan melanogenesis ditentukan dengan menggunakan kaedah pemendapan western. Dalam kajian ini, kulit melanosit telah dirawat dengan kepekatan air kelapa muda yang berbeza dan dibanding dengan sel-sel tidak dirawat. Hasil dari asai MTT menunjukkan bahawa air kelapa muda mempamerkan tiada kesitotoksikan pada melanosit di 10 mg / ml dan kepekatan kesitotoksikan setengah maksimal (CC_{50}) ialah 13.12 mg / ml. Kajian Kajian ini menunjukkan bahawa air kelapa muda mengurangkan aktiviti tirosinase oleh merencat aktivitinya dengan IC_{50} (kepekatan setengah maksimal rencatan) nilai 10 mg / ml dan juga turun pengaturan tahap protein tirosinase. Keputusan daripada pemendapan

western menunjukkan bahawa tahap protein tirosinase-2 menurun secara signifikan pada 8 dan 10 mg / ml air kelapa muda masing-masing dengan 0.351 dan 0.280 kali ganda. Tiada pengurangan yang signifikan dalam tahap protein tirosinase-1 (TRP-1). Selain itu, air kelapa muda pada kepekatan yang rendah tidak mengurangkan aktiviti tirosinase secara signifikan. Kesimpulannya, air kelapa muda pada 8 dan 10 mg / ml jelas mengurangkan tahap protein tirosinase dan protein tirosinase-2 (TRP-2) dengan lebih 80% kebolehhidupan. Keputusan ini menunjukkan bahawa air kelapa muda mungkin dianggap sebagai agen pemutihan yang berpotensi dalam kosmetik.

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

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
APS	Ammonium per sulfate
AHA	α-hydroxyacid
B1	Thiamim
B2	Riboflavin
B3	Niacin or Niacinamide
B5	Pantothenic acid
B6	Pyridoxine
B7	Biotin
B9	Folic acid
BPE	Bovine pituitary extract
$CaCl_2$	Calcium chloride
CC_{50}	Half maximal cell cytotoxicity
DHICA	5, 6-dihydroxyindole-2-carboxylic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
rhFGF-B	rh fibroblast growth factor-B
GAs	Gibberellins
GA	Gentamicin sulfate & Amphotericin-B
HQ	Hydroquinone
HepG2	Human hepatoma cell line
IC_{50}	Half maximal inhibitory concentration
L-DOPA	Dihydroxyphenylalanine
MAWA	Malaysian Red Dwarf" mother & "West African Tall
mT	Milli-Torr
α-MSH	Melanocyte-stimulating hormun
MITF	Microphthalmia-associated Transcription Factor
MAPK	Mmitogen-activated protein kinase
MGM-4	Melanocyte growth medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHEM-neo	Neonatal normal human epidermal melanocyte

OD	Optical density
PMA	Phorbol 12-myristate 13-acetate
PBS	Phosphate-buffered saline
PIH	Post Inflammatory Hyperpigmentation
PAR-2	Protease Activated Receptor-2
PAK	Phenylalanine hydroxylase
PKA	Protein kinase A
POMC	Proopiomelanocortin
rh-insulin	Rrecombinant human insulin
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
TYR	Tyrosinase
TRP-1	Tyrosinase related protein-1
TRP-2 (DCT)	Tyrosinase related protein-2 (Dopachrome tatumerase)
TNS	Trypsin neutralizing solution
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween
TCW	Tender Coconut Water
TH1	Tyrosine hydroxylase isoenzyme 1
UV	Ultraviolet
UPM	Universiti Putra Malaysia
YCW	Young Coconut Water

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Today, a variety of cosmetics and clinical products are specially used in Asian countries to balance the colour of skin without making undesirable changes on the skin such as irritation or abnormal pigmentation. Since some of whitening agent are expensive and unsafe, the cosmetic factories are looking for safe, efficient and novel cosmetic agents. These products are tested on human skin to examine their effect on the melanogenesis. Hence, to investigate and evaluate the effect of whitening agents, the pigment producing cells such as melanocytes are being used (Costin & Raabe, 2013).

Melanocytes are dendritic cells that located in deepest layer of skin. The colour of the human skin is determined by type, size, amount, and deposition of melanin pigment in the melanocyte cells. The melanogenesis is enhanced by exposure of skin to UV radiation and also by activation of the melanogenesis enzyme, tyrosinase (Momtaz *et al*, 2008; Gillbro & Olsson, 2011). Tyrosinase is a bifunctional copper- containing enzyme and involves two different catalytic reactions: first, tyrosinase hydroxylates the L-tyrosine to the dihydroxyphenylalanine (L-DOPA) and then converts L-DOPA to dopaquinone. In series of non-enzymatic reactions, dopaquinone is converted to the dopachrome (Baurin *et al*., 2002). There are two types of melanin pigments: eumelanin (a dark brown-black pigment) and pheoumelanin (a light red-yellow pigment). Besides, tyrosinase (Poole *et al*., 2013), tyrosinase related protein 1 (TRP-1) and tyrosinase related protein 2 (TRP-2) also known as dopachrometatumerase (DCT), are involved in melanogenesis. Dopachrome is spontaneously transformed to eumelanin or by TRP-2, enzymatically converted to 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) to form eumelanin. The TRP-1 increases tyrosinase stability and the ratio of eumelanin to pheomelanin. The pheomelanin pathway which is determined by the presence of cysteine, is required to convert dopaquinone to cysteinyl-dopa in order to form pheomelanin. Tyrosinase inhibitors might supress melanin production in the melanocyte (Gillbro & Olsson, 2011; Ebanks *et al*., 2009).

Recently, several melanogenesis inhibitors are used in the cosmetic and pharmaceutical companies as skin-whitening agents (Hanamura *et al*., 2008). Many of skin whitening agents decrease the total melanin production. Nowadays, some of these skin-lightening inhibitors such as kojic acid, arbutin (Maeda & Fukuda, 1991) and hydroquinone (Jimbow *et al*., 1974) are used in cosmetics and beauty products (Gillbro & Olsson, 2011).

Coconut is the coconut palm fruit botanically known as *Cocos nucifera.*, and grows in tropical regions like Malaysia, Indonesia and India. The coconut water is widely consumed as refreshing beverage in the world especially in tropical areas because it is nutritious and provides health benefits for the body (Yong *et al*., 2009). It was reported that coconut water has a lightening effect on hyperpigmentation such as melanoma. The study showed that coconut water can reduce melanin production on the mouse melanoma (S91 cell line) by reducing the enzymes involved in melanogenesis pathway such as TRP-2 enzyme (Mahalingam *et al*., 2009).

In Malaysia “Mawa” is considered as high quality and unique coconut that is used for producing food and beverage product. Mawa coconut is cross-hybrid fruit, originated from “Malaysian Red Dwarf” mother and “West African Tall”. Among natural beverages in tropical regions, coconut water has huge potential commercialization industry (Pau & Chan, 1985). In this study, to examine cytotoxic effect of young coconut water (YCW) on skin cells (melanocytes), different concentrations of the extract were tested on neonatal normal human epidermal melanocytes (NHEM-neo).

1.2 Problem Statement

Since, many skin-whitening agents are used for treating and preventing hyperpigmentation disorders in cosmetics industries, it is important to consider the issue of safety of these lightening-agents on human skin (Kim *et al*., 2013b). Some depigmenting agents such as hydroquinone (HQ), kojic acid and arbutin are widely used in cosmetic products. It was found that some of these lightening agents such as HQ produces a lot of side effects namely, skin irritation and destruction of melanocytes of the human skin. To avoid the risk of mutagenesis, HQ is prohibited in cosmetics production by European Union and United States. Other skin whitening agents such as kojic acid and arbutin have limited efficacy due to poor skin penetration and instability (Ubeid *et al*., 2009).

Therefore, it is a main challenge for the cosmetics industries to apply whitening agents with less side effects. Thus, it is necessary to find natural lightening agents with more efficacy and less cytotoxicity effect on human skin. Whitening cosmetics are products with high consumption and daily usage, especially in tropical regions. Hence, the natural, inexpensive and achievable whitening resources are in demand. In this study, the effect of YCW on melanogenesis of human skin cells was undertaken to investigate its whitening effect.

1.3 Objectives

General Objective

- To investigate the effect of young coconut water on melanogenesis using neonatal normal human epidermal melanocyte

Specific Objectives

- To determine the cytotoxicity of young coconut water on neonatal normal human epidermal melanocyte using MTT assay.
- To determine the effect of young coconut water on cellular tyrosinase activity in neonatal normal human epidermal melanocyte.
- To determine melanin content of neonatal normal human epidermal melanocyte treated with young coconut water.
- To access changes in protein level of tyrosinase, tyrosinase related protein-1 and tyrosinase related protein-2 known as dopachrome tautamerase in neonatal normal human epidermal melanocyte treated with young coconut water using western blot technique.

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