UNIVERSITI PUTRA MALAYSIA

INHIBITION OF MELANOGENIC ACTIVITY BY CHALCONE DERIVATIVES IN ALPHA-MELANOCYTE STIMULATING HORMONE CELL LINE (B16-F10)

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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October 2014

Chairman: **Syahida Ahmad, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Hyperpigmentation or dark patches on skin have been increasingly reported over past few decades. Overproduction of melanin by irregular melanogenesis due to high exposure of ultraviolet contributes to many aesthetic problems. Excessive exposure to ultraviolet radiation causes elevation of alpha-melanocyte stimulating hormone (α-MSH) production leading to undesired pigmentation process. Whitening and bleaching agents are among the therapeutic choices in treating hyperpigmentation. However, some of these whitening agents such as kojic acid and hydroquinone were claimed to exhibit detrimental effects while others such as arbutin and ascorbic acid demonstrated low efficacy as depigmenting agent. Thus, alternative therapeutics preferences were derived from natural products in effort to provide safe yet reliable depigmenting agents. Chalcone and its derivatives have been reported to have pharmaceutical effect of depigmenting activity. In the present study, ten chalcone derivatives were screened for anti-tyrosinase activity using mushroom tyrosinase assay. Effects of selected chalcone derivatives on cellular melanin production as well as tyrosinase activity were evaluated in α-MSH-stimulated B16-F10 cells. The chalcone derivatives were further elucidated for melanogenic genes expressions of *Tyr*, *Trp-1*, *Trp-2* and *Mitf*. Out of ten compounds, seven demonstrated promising anti-tyrosinase activity which were 3-(4-Amino-phenyl)-1-(4-hydroxy-phenyl)-propenone (AQ), 1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-phenyl-propenone (FLB), 1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-(4-methoxy-phenyl)-propenone (FLA), 1-(2,4-Dihydroxy-phenyl)-3-(2,3-dimethoxy-phenyl)-propenone (E-5), 3-(3,4-Dihydroxy-phenyl)-1-(2-hydroxy-4,6-dimethoxy-phenyl)-propenone (E-8), 3-(4-Chloro-phenyl)-1-(2,4-dihydroxy-phenyl)-propenone

(EY-1) and 1-(5-Chloro-2-hydroxy-phenyl)-3-(3,4-dimethoxy-phenyl)-propenone (D-32) with IC_{50} values of 15.95 ± 0.83μM, 15.74 ± 1.92 μM, 17.22 ± 1.21 μM, 17.70 ± 1.04 μM, 21.39 ± 1.12 μM, 28.18 ± 1.74 μM and 46.99 ± 2.54 μM respectively. Accordingly, toxicity effects of the potential chalcone derivatives were evaluated on α-MSH-stimulated B16-F10 cells using MTT assay whereby only FLA and FLB showed lowest cytotoxic effect with 82.25 ± 1.52 % and 80.41 ± 0.78 % of cell viability respectively. Reducing effects towards melanin content and cellular tyrosinase activity in α-MSH-stimulated B16-F10 cells indicated that FLA significantly reduced the specific cellular melanin content in cells by 7-fold (0.48 ± 0.04 μg melanin/μg protein) and FLB by12-fold (0.28 ± 0.04 μg melanin/μg protein). Specific cellular tyrosinase activity was inhibited by FLA and FLB by 11-fold (0.74 ± 0.04 μU/μg protein) and 20-fold (0.42 ± 0.02 μU/ μg protein) respectively. At molecular level, treatments of FLA and FLB suppressed all melanogenic genes expressions of *Tyr*, *Trp-1*, *Trp-2* and *Mitf* in α-MSH-stimulated B16-F10 cells. Interestingly, at the highest concentration of 50 μM tested, both FLA and FLB showed highest suppression on *Tyr* gene by 20-fold (0.05 ± 0.01 fold expression) and 50-fold (0.02 ± 0.01 fold expression) respectively. Findings from the study have provided mechanistic insights for the depigmenting actions of chalcone derivatives on α-MSH-stimulated B16-F10 cells via suppression of melanogenic genes of *Tyr*, *Trp-1*, *Trp-2* and *Mitf*. With these results, it could be extrapolated that by limiting the melanogenic responses of B16-F10 cells, the melanin production as well as tyrosinase activity associated with hyperpigmentation may be lessened by FLA and FLB. Thus, both chalcone derivatives could be used as lead compounds on developing new depigmenting agents.

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

PERENCATAN AKTIVITI MELANOGENIK OLEH DERIVATIF KALKON DALAM TITISAN SEL HORMON PERANGSANG ALFA-MELANOSIT (B16-F10)

Oleh

NURSHAFIKA BINTI MOHD SAKEH

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Hiperpigmentasi atau tompokan gelap pada kulit telah menunjukkan peningkatan sejak beberapa dekad yang lalu. Lebihan pengeluaran melanin oleh melanogenesis yang tidak teratur kesan daripada pendedahan tinggi radiasi ultraungu telah terbukti menyumbang kepada banyak masalah estetik. Pendedahan berlebihan kepada radiasi ultraungu akan menyebabkan peningkatan pengeluaran hormon perangsang alfa-melanosit (α-MSH) membawa kepada proses pigmentasi yang tidak dikehendaki. Ejen pemutih dan peluntur adalah antara pilihan rawatan dalam mengubati hiperpigmentasi. Walaubagaimanapun, sesetengah ejen pemutih seperti asid kojik dan hidrokuinon didakwa menyebabkan kesan merbahaya sementara yang lainnya seperti arbutin dan asid askorbik menunjukkan kesan yang lemah sebagai ejen depigmentasi. Maka, beberapa pilihan alternatif terapeutik telah diperolehi daripada produk asli dalam usaha menyediakan ejen depigmentasi yang selamat bahkan boleh diharapkan. Kalkon dan derivatifnya telah dilaporkan mempunyai kesan farmaseutikal dalam aktiviti depigmentasi. Dalam kajian terkini, sepuluh derivatif kalkon telah disaring sebagai anti-tirosinase menggunakan esei tirosinase cendawan. Efek kalkon derivatif terpilih terhadap penghasilan melanin dan aktiviti tirosinase dalam sel B16-F10 yang dirangsang α-MSH telah dinilai. Derivatif kalkon dinilai bagi ekspresi gen melanogenik *Tyr*, *Trp-1*, *Trp-2* dan *Mitf* . Daripada sepuluh kompaun, tujuh daripadanya telah menjanjikan aktiviti anti-tirosinase iaitu 3-(4-Amino-phenyl)-1-(4-hydroxy-phenyl)-propenone (AQ), 1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-phenyl-propenone (FLB), 1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-(4-methoxy-phenyl)-propenone (FLA), 1-(2,4-Dihydroxy-phenyl)-3-(2,3-dimethoxy-

phenyl)-propenone (E-5), 3-(3,4-Dihydroxy-phenyl)-1-(2-hydroxy-4,6-dimethoxy-phenyl)-propenone (E-8), 3-(4-Chloro-phenyl)-1-(2,4-dihydroxy-phenyl)-propenone (EY-1) dan 1-(5-Chloro-2-hydroxy-phenyl)-3-(3,4-dimethoxy-phenyl)-propenone (D-32) dengan nilai IC_{50} 15.95 ± 0.83µM, 15.74 ± 1.92 µM, 17.22 ± 1.21 µM, 17.70 ± 1.04 µM, 21.39 ± 1.12 µM, 28.18 ± 1.74 µM dan 46.99 ± 2.54 µM masing-masing. Sewajarnya, kesan toksik derivatif kalkon yang berpotensi telah dikaji terhadap sel B16-F10 yang dirangsang α-MSH menggunakan esei MTT yang mana hanya FLA dan FLB menunjukkan tahap toksik kepada sel yang paling rendah dengan 82.25 ± 1.52 % dan 80.41 ± 0.78 % sel hidup masing-masing. Efek pengurangan terhadap kandungan melanin dan aktiviti tirosinase dalam sel B16-F10 yang dirangsang α-MSH menunjukkan FLA telah mengurangkan dengan signifikan kandungan spesifik melanin dalam sel MSH dengan 7-kali ganda (0.48 ± 0.04 µg melanin/µg protin) dan FLB dengan 12-kali ganda penurunan (0.28 ± 0.04 µg melanin/µg protin). Aktiviti spesifik tirosinase dalam sel telah direncatkan oleh FLA dan FLB dengan 11-kali ganda (0.74 ± 0.04 µU/µg protin) dan 20-kali ganda (0.42 ± 0.02 µU/ µg protin) masing-masing. Dalam tahap molekul, rawatan oleh FLA dan FLB menyekat semua ekspresi gen *Tyr*, *Trp-1*, *Trp-2* dan *Mitf* dalam sel B16-F10 yang dirangsang α-MSH. Menariknya, pada kepekatan tertinggi 50 µM, kedua-dua FLA dan FLB telah menunjukkan sekatan tertinggi terhadap gen *Tyr* dengan 20-kali ganda (0.05 ± 0.01 kali ganda ekspresi) dan 50-kali ganda (0.02 ± 0.01 kali ganda ekspresi) masing-masing. Keputusan kajian ini telah menyediakan pendedahan terhadap mekanisma aktiviti depigmentasi oleh derivatif kalkon terhadap sel B16-F10 yang dirangsang α-MSH melalui perencatan gen melanogenik *Tyr*, *Trp-1*, *Trp-2* dan *Mitf*. Melalui keputusan ini, ia boleh dicadangkan bahawa dengan menghadkan respon melanogenik sel B16-F10, pengeluaran melanin begitu juga aktiviti tirosinase yang berkait langsung dengan hiperpigmentasi boleh dikurangkan dengan FLA dan FLB. Oleh itu, kedua-dua dervatif kalkon boleh digunakan sebagai peneraju kompaun untuk membangunkan ejen depigmentasi baharu.

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I certify that a Thesis Examination Committee has met on 29th October 2014 to conduct the final examination of Nurshafika binti Mohd Sakeh on her thesis entitled " Inhibition of Melanogenic Activity by Chalcone Derivatives in Alpha-Melanocyte Stimulating Hormone Cell Line (B16/F10)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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 follow:

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This to confirm that:

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Signature: _____	Signature: _____
Name of Chairman of Supervisory Committee: Syahida Ahmad	Name of Member of Supervisory Committee: Zetty Norhana Balia Yusof
Signature: _____	
Name of Member of Supervisory Committee: Muhammad Nadeem Akhtar	

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AC	adenylate cyclase
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AR	Arbutin
ASP	Agouti signal protein
ATCC	American Type Culture Collection
AQ	3-(4-Amino-phenyl)-1-(4-hydroxy-phenyl)-propenone
bHLH-LZ	basic-helix-loop-helix-leucine-zipper
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cAMP	cylic adenosine monophosphate
cDNA	complementary Deoxyribonucleic acid
CO_2	Carbon dioxide
CREB	cAMP-responsive element binding
C_T	Threshold cycle
D-31	1-(5-Chloro-2-hydroxy-phenyl)-3-(4-methoxy-phenyl)-propenone
D-32	1-(5-Chloro-2-hydroxy-phenyl)-3-(3,4-dimethoxy-phenyl)-propenone
DHI	Dihydroxyindole
DHICA	Dihydroxyindole carboxylic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine
dsDNA	double strand DNA
E	Efficiency
E-5	1-(2,4-Dihydroxy-phenyl)-3-(2,3-dimethoxy-phenyl)-propenone
E-8	3-(3,4-Dihydroxy-phenyl)-1-(2-hydroxy-4,6-dimethoxy-phenyl)-propenone
EDTA	Ethylenediaminetetraacetic acid
EY-1	3-(4-Chloro-phenyl)-1-(2,4-dihydroxy-phenyl)-propenone
EY-6	3-(4-Amino-phenyl)-1-(2-hydroxy-4,6-dimethoxy-phenyl)-propenone
EY-7	3-(4-Amino-phenyl)-1-(2,4,6-trimethoxy-phenyl)-propenone
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLA	1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-(4-methoxy-phenyl)-propenone
FLB	1-(2-Hydroxy-4,6-dimethoxy-phenyl)-3-phenyl-propenone
GAPDH	Glyceraldehyde 3‑phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
HQ	Hydroquinone
h	hour/s
IC_{50}	Inhibitory concentration 50%
Ig	Immunoglobulin

IR	Infrared
KA	Kojic acid
kb	kilobase
kDa	kiloDalton
kHz	kiloHertz
L	Litre
µg	Microgram
µL	Microlitre
µM	Micromolar
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MC1R	Melanocortin 1 receptor
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MITF / *Mitf*	Microphthalmia transcription factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
α-MSH	alpha-melanocyte stimulating hormone
MW	Molecular weight
NaOH	Sodium hydroxide
NCBI	National Centre of Biotechnology Information
nM	Nanomolar
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NTC	Non-template control
PAR-2	Protein-activated receptor-2
PBS	Phosphate buffer saline
PKA	Protein kinase A
PMSF	Phenylmethanesulfonyl fluoride
POMC	Propriomelanocortin
RER	Rough endoplasmic reticulum
ROS	Reactive Oxygen Species
RNA	Ribonucleic acid
rpm	Rotation per minute
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
s	second/s
SCF	Stem cell factor
SEM	Standard error of mean
TRPs	Tyrosinase related proteins family
TRP-1/ *Trp-1*	Tyrosinase-related protein 1
TRP-2/ *Trp-2*	Tyrosinase-related protein 2
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tyr / *Tyr*	Tyrosinase
U	Unit
UV	Ultraviolet
V	Voltan

WS4	Waarderburg-Hirschsprung
α	Alpha
x g	Gravities (Unit for relative centrifugal force)
^{o}C	Degree celcius

CHAPTER 1

INTRODUCTION

Increasing aesthetic concerns among society has tremendously affected people worldwide. Abnormal variations in skin color due to hyperpigmentation problems do not only constitute a cosmetic liability but also in many instances results in formidable emotional and social problems (Balkrishnan et al., 2004). Skin pigmentation disorders may be associated with dysfunction of pigment-producing cells. Uncontrolled hyperproliferation of melanocytes can trigger the initiation of melanoma, which is the most aggressive skin cancer, mainly due to its high metastatic potential (Kormos, 2011). Hyperpigmentation has also been closely associated with excessive exposure of ultraviolet (UV) radiations. Exposure to UV radiations with specific wavelengths 320-400 nm (UVA), 280-320 nm (UVB) and 200-280 nm (UVC) may cause serious health issues (Agar and Young, 2005). While UVC is effectively filtered by stratospheric ozone from reaching the earth's surface, UVA and UVB radiations may both reach living organisms resulting in biological alterations to the skin and eyes (Matsumura and Ananthaswamy, 2003). UV exposure results in formation of free radicals, reactive oxygen species (ROS) and may cause mutations to genomic stability of DNA by dimerization of thymine nucleotides which ultimately can lead to skin cancers (Brenner and Hearing 2007; Slominski et al., 2004).

During melanogenesis, melanocytes in skin play a crucial role as the site of melanin synthesis. In the normal pigmentation process, melanin is transported to the apical face of keratinocytes nuclei providing protection towards DNA from UV damage (Tsatmali et al., 2002). However, in the case of excessive stimulation of melanogenesis, it causes severe pigmentation abnormalities. Melanin synthesis itself results in production of toxic free radicals such as hydrogen peroxide and quinone intermediates (Meyskens et al., 2001). Severity of the effect is dependent on melanin types wherein eumelanin causes hyperpigmentation whilst pheomelanin induces phototoxicity which is linked to skin cancers (Meierjohann, 2013; Wenczl et al., 1998). It is reported that individuals with high levels of pheomelanin and low eumelanin levels are prone to be UV sensitive (Praetorius et al., 2014; Vincensi et al., 1998). According to World Health Organization (WHO, 2009), 50% to 90% of reported cases of skin cancers are due to UV radiation. However, abnormalities of pigmentation can also be contributed by chronic inflammation, mechanical trauma of skin and irregular α-MSH release (Ortonne and Nordlund, 2007).

At transcriptional level, microphthalmia-associated transcription factor (MITF) has been identified as the master regulator of melanogenesis which regulates tyrosinase related proteins family (TRPs) namely tyrosinase, tyrosinase-related protein 1 and 2 (TRP-1 and TRP-2) (Aksan and Goding, 1998; Bertolotto et al., 1998). Ever since tyrosinase is

classified as rate-limiting enzyme in melanin synthesis, numerous research efforts have been done by researchers in order to combat the hyperpigmentation issues. Whitening agents such as kojic acid and hydroquinone have been reported to effectively treat hyperpigmentation (Goncalez et al., 2013; Cabanes et al., 1994). Safety assessment of these two drugs has provoked great health concerns. Kojic acid was reported to enhance hepatocarcinogenesis (Takizawa et al., 2004) while hydroquinone was later reported to result in genotoxic effects through oxidative DNA damage in animal models (Luo et al., 2008). Thus, long-term usage of these two whitening agents has therefore been prohibited in several countries. On the other hand, arbutin and ascorbic acid were not toxic but demonstrated weak whitening effect (Maeda and Fukuda, 1996). Thus, the discovery of alternative anti-pigmenting agents or treatments is of utmost important.

On natural preference of the treatment, a wide spectrum of phytochemicals and their derivatives have been identified for development as depigmenting agents (Kim et al., 2007; Shin et al., 1998; Yokota et al., 1998). Interestingly, chalcone and its derivatives which are widely present as secondary metabolite in plants have been recognized potent tyrosinase inhibitors (Zhang et al., 2009; Jun et al., 2007; Nerya et al., 2004). Recently, lots of studies have been conducted to explain the structure-activity relationship (SAR) of chalcones in providing better inhibitory mechanisms towards tyrosinase enzyme (Khatib et al., 2007; Kim et al., 2006; Nerya et al., 2004). The findings have enormously encouraged the development of better amendment of newly synthesized chalcones.

Hyperpigmentation-related diseases pose aesthetic issues that may be addressed by the use of anti-melanogenic agents. A target of these agents is tyrosinase, the rate limiting enzyme in melanin synthesis. Here, a series of chalcone derivatives were first screened for inhibitory activities against mushroom tyrosinase. Selected samples were then further tested for their effects on melanin production, tyrosinase activity and expression of melanogenic genes, *Tyr*, *Trp-1*, *Trp-2* and *Mitf* in α-MSH-stimulated B16-F10 cells. In the present study, α-MSH stimulation was manipulated on cells in order to mimic hyperpigmentation problems due to over-exposure of UV radiation.

The study tests hypothesis that chalcone derivatives might demonstrate not only inhibition towards cellular melanin production and tyrosinase activity in α-MSH-stimulated B16-F10 cells by suppressing *Mitf* gene, which in turn down-regulated *Tyr*, *Trp-1* and *Trp-2* genes expressions.

Objectives of study

The general objective of this study is to elucidate the anti-melanogenic effects of chalcone derivatives in α-MSH-stimulated B16-F10 melanoma cells.

The specific objectives are:

1. To screen chalcone derivatives with anti-tyrosinase activity.
2. To evaluate effects of selected chalcone derivatives on melanin production and tyrosinase activity in α-MSH-stimulated B16-F10 melanoma cells.
3. To determine effects of selected chalcone derivatives on genes expressions of *Tyr*, *Trp-1*, *Trp-2* and *Mitf* in α-MSH-stimulated B16-F10 melanoma cells.

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