ฤทธิ์ของแซนโทไซลินต่อการสร้างเม็ดสีผิว

นางสาว วันใหม่ โมฬีพันธ์

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EFFECT OF XANTHOXYLIN ON MELANOGENESIS

Miss Wanmai Moleephan

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By	Miss Wanmai Moleephan
Field of Study	Pharmacology
Thesis Advisor	Associate Professor Supeecha Wittayalertpanya
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

…….. Thesis Advisor
(Associate Professor Supeecha Wittayalertpanya)

…….. Thesis Co-Advisor
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

…….. Examiner
(Assistant Professor Naowarat Suthamnatpong, Ph.D.)

…….. External Examiner
(Associate Professor Wannasri Sindhuphak, M.D.)

……External Examiner
(Assistant Professor Panida Vayumhasuwan, Ph.D.)

วันใหม่ โมฬีพันธ์ : ฤทธิ์ของแซนโทไซลินต่อการสร้างเม็ดสีผิว. (EFFECT OF XANTHOXYLIN ON MELANOGENESIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. สุพีชา วิทยเลิศปัญญา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร. นิจศิริ เรืองรังษี, 89 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของแซนโทไซลินซึ่งเป็นสารฟีนอลิกที่พบในพืชหลายชนิดรวมทั้ง *Zanthoxylum piperitum* ต่อการสร้างเม็ดสีผิวในเซลล์เมลาโนไซต์ของหนู คือ เซลล์ B16F10 แซนโทไซลินที่ความเข้มข้น 6.25, 12.5 และ 25 ไมโครกรัม/มิลลิลิตร เหนี่ยวนำให้มีการสร้างเม็ดสีผิวเพิ่มขึ้นตามความเข้มข้นของสารโดยไม่เป็นพิษต่อเซลล์ เพิ่มจำนวนและความยาวของเดนไดรต์ของเซลล์ การสร้างเม็ดสีผิวส่วนใหญ่ถูกควบคุมโ ดย MITF ที่เป็น transcription factor สำคัญที่ควบคุมการแสดงออกของ tyrosinase-related protein 1 (TRP-1) และ tyrosinase-related protein 2 (TRP-2) และ tyrosinase ที่เป็นโปรตีนและเอนไซม์สำคัญในการสร้างเม็ดสีผิวในเมลาโนโซมของเซลล์เมลาโนไซต์ การศึกษากลไกการออกฤทธิ์ของแซนโทไซลินต่อการสร้างเม็ดสีผิว พบว่า แซนโทไซลินที่ความเข้มข้น 6.25, 12.5 และ 25 ไมโครกรัม/มิลลิลิตร เหนี่ยวนำให้เพิ่มการแสดงออกในระดับ mRNA ของ MITF และ tyrosinase แต่ไม่มีผลต่อการแสดงออกของ TRP-1 และ TRP-2 การกระตุ้น protein kinase A (PKA) ผ่านทาง cAMP เป็นวิถีทางหลักในการควบคุมการแสดงออกของ MITF และ tyrosinase นอกจากนี้การสื่อสัญญาณผ่าน Phosphatidylinositol-3-Kinase (PI3K)-mediated (PKB), PKC, and MAP kinase (MAPK) พบว่ามีบทบาทต่อการแสดงออกของโปรตีนสองตัวนี้ได้เช่นกัน การให้แซนโทไซลินที่ความเข้มข้น 25 ไมโครกรัม/มิลลิลิตร ร่วมกับตัวยับยั้งของ protein kinase ที่กล่าวมาแล้วที่ความเข้มข้น 5 ไมโครโมลาร์ พบว่า ตัวยับยั้ง protein kinase เหล่านี้ทำให้การเหนี่ยวนำให้เซลล์สร้างเม็ดสีผิวของแซนโทไซลินลดลง โดยมีความแรงในการยับยั้งเป็นดังนี้ ตัวยับยั้ง PKA > PKC > MAPK (MEK1) = PKB ตัวยับยั้ง PKA และ PKC ยังมีผลยับยั้งฤทธิ์ของแซนโทไซลินในการเพิ่มการแสดงออกในระดับ mRNA ของ MITF และ tyrosinase ขณะที่ตัวยับยั้ง PKB และ MEK1 ไม่มีผลต่อการออกฤทธิ์ของสาร โดยตัวยับยั้ง PKA ยับยั้งได้มากกว่าตัวยับยั้ง PKC ผลจากการศึกษานี้แสดงว่า แซนโทไซลินกระตุ้นการสร้างเม็ดสีผิวในเซลล์เมลาโนไซต์ B16F10 โดยเพิ่มการแสดงออกของ MITF และ tyrosinase โดยอาจมีวิถีทางหลัก คือ การกระตุ้น PKA ผ่านทาง cAMP การสื่อสารผ่านทางวิถีทางอื่นอาจมีบทบาทในการออกฤทธิ์ของแซนโทไซลินได้เช่นกัน

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5087193220 : MAJOR PHARMACOLOGY
KEYWORDS : XANTHOXYLIN/ MELANOGENESIS/ MELANIN/ TYROSINASE

WANMAI MOLEEPHAN : EFFECT OF XANTHOXYLIN ON MELANOGENESIS. THESIS ADVISOR : ASSOC. PROF. SUPEECHA WITTAYALERTPANYA, THESIS CO-ADVISOR : ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 89 pp.

This study intended to investigate the effect of xanthoxylin, a phenolic compound found in many plants as well as *Zanthoxylum piperitum*, on melanin production in mouse melanoma B16F10 cells. Xanthoxylin at the concentrations 6.25, 12.5 and 25 μg/ml significantly induced melanin production in B16F10 cells in a concentration dependent manner without any effect on cell viability. It also increased number of dendrites per cell and induced the elongation of dendrites of these melanoma cells. The mechanisms of action of xanthoxylin on melanogenesis in B16F10 cells were also investigated. MITF is a critical transcription factor that regulates the expression of tyrosinase-related protein 1 (TRP-1), tyrosinase-related protein 2 (TRP-2) and tyrosinase which are proteins or enzymes associated with melanin synthesis in melanosome of melanocytes. Xanthoxylin at the concentrations 6.25, 12.5 and 25 μg/ml significantly induced the mRNA expression of MITF as well as tyrosinase. However it did not change the mRNA expression of TRP-1 and TRP-2. Cyclic AMP-mediated protein kinase A (PKA) activation is the major signaling pathway for MITF and tyrosinase expression. Phosphatidylinositol-3-Kinase (PI3K)-mediated (PKB), PKC, and MAP kinase (MAPK) signaling pathways are the other pathways that also regulate the expression of MITF and tyrosinase. By treating B16F10 cells with 25 μg/ml xanthoxylin and 5 μM specific inhibitors of these protein kinases, these inhibitors significantly decreased xanthoxylin-induced melanin production in the following order of potency; inhibitors of PKA > PKC > MAPK (MEK1) = PKB. The inhibitors of PKA and PKC also significantly decreased xanthoxylin-stimulated MITF and tyrosinase mRNA expression while PKB and MAPK did not have any effect on this xanthoxylin activity. The inhibitory activity of the PKA inhibitor was higher than the PKC one. The results from this study suggest that xanthoxylin stimulated melanogenesis in B16F10 cells by up-regulating MITF and tyrosinase expression. It is possible that xanthoxylin may activate melanin production mainly via cAMP-mediated PKA activation. Other signaling pathways may also play role in xanthoxylin-induced melanogenesis.

Field of Study : Pharmacology Student's Signature .....
Academic Year : 2009 Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ATCC	American Type Cell Culture
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CBP	CREB binding protein
CO_2	carbon dioxide
CRE	cAMP response element
CREB	cAMP response element-binding protein
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
h	hour
HCl	hydrochloric acid
IC50	The half maximal inhibitory concentration
kg	kilogram(s)
mg	milligram(s)
ml	milliliter(s)
mm	millimeter(s)
mRNA	messenger ribonucleic acid
M	molar (mole per liter)
MITF	microphthalmia-associated transcription factor
ng	nanogram(s)
nm	nanometer
nM	nanomolar
NaCl	sodium chloride
NaOH	sodium hydroxide

OD	optical density (absorbance)
pH	the negative logarithm of hydrogen ion concentration
PBS	phosphate buffer saline solution
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
rpm	revolution per minute
RNA	ribonucleic acid
S.E.M	standard error of the mean
TNF-α	tumor necrosis factor-alpha
UV	ultraviolet
v	volume(s)
^{0}C	degree Celsius
µg	microgram(s)
µl	microliter(s)
µmol	micromole(s)
α-MSH	alpha-melanocyte stimulating hormone

CHAPTER I

INTRODUCTION

Background and Rationale

Melanin is a complex of insoluble eumelanin and pheomelanin monomers that responsible for skin pigmentation [1]. It resides in both melanocyte and keratinocyte, known as the epidermal melanin unit. Melanin synthesis is regulated by tyrosinase, tyrosinase-related protein (TRP)-1 and (TRP)-2, and their crucial transcription factor microphthalmia-associated transcription factor (MITF) [2, 3]. Tyrosinase is a key enzyme in the rate limiting step of melanin synthesis [4, 5]. After synthesis, melanin is deposited in specific cellular organelles called melanosomes and transferred via dendrites to the surrounding keratinocytes. Within melanocytes or keratinocytes melanin often accumulates as a nuclear cap [2]. The important role of melanin is protection against UV from sunlight which induced DNA damage in human skin by absorbing and scattering UV radiation [3]. The rate of melanin synthesis and the transfer of melanin via melanocyte dendrites are influenced by races, ultraviolet light exposure and other stimuli [6]. It has been reported that light-skinned individuals are highly susceptible to sun-induced skin cancers and skin aging [7]. Tanning is desirable by some persons, especially European and North American people [8]. The most common way of darkening skin is sun-tanning, using either natural sunlight or ultraviolet light sources. However, prolonged exposure of human skin to ultraviolet light is known to have long and short term adverse effects, including the risk of painful sunburn and keratitis in a short term exposure, and skin aging or skin cancer for long term exposure [9].

The loss of skin pigmentation or hypopigmentation is the result of either a reduction in melanin production or decreased numbers or absence of melanocytes in the epidermis [10]. Examples of hypopigmentation include vitiligo which causes smooth, white patches on the skin, albinism which is an inherited disorder caused by the absence of an enzyme that produces melanin, and the most common hypopigmentation as a result of skin damage from a skin infection, blisters, burns or other trauma [11].

A limited treatment options with the goal of restoring melanin pigment are available including topical corticosteroids [11], narrow-broad band UVB [12], and PUVA (psoralen plus UVA) [13, 14, 15]. Of the skin pigmentation enhancers, PUVA and melanocyte stimulating hormone (MSH) analogs have been clinically used under safety and side effect consideration [13]. Several agents, from natural sources or synthetic compounds, have been reported to enhance skin pigmentation including L-tyrosine which is precursor of melanin synthesis, L-dihydroxyphenylalanine (L-Dopa) and L-dopa phosphates, lysosomotropic agents such as ammonium chloride, diacylglycerols (DAG), pTpT and DNA fragments, melanocyte stimulating hormone (MSH) and peptide analogs, 3-Isobutyl-1-methylxanthine (IBMX), nitric oxide (NO) donors, bicyclic monoterpene (BMT) diols [13]. Some of these agents are used as cosmetics to simulate natural melanin such as dihydroxyacetone (DHA) and topical melanins [13, 16]. To date, no successful therapy exists to safely repigmentation in hypopigmentation. Investigation for safe and effective skin pigmentation stimulators is still needed.

Several phenolic compounds from natural sources have been reported to stimulate melanin synthesis [16]. Xanthoxylin is also a phenolic compound found in several plants such as *Zanthoxylum piperitum* and *Sebastiana schottiana*. Several activities of xanthoxylin have been demonstrated including antagonistic effect against several neurotransmitter-mediated contractions in nonvascular smooth muscles [17], antifungal [18], antispasmodic [19], antioedema [20] and inhibitor of prostaglandin synthetase and 5-lipoxygenase, cytotoxic against Ehrlich ascites tumour cells [21]. Due to phenolic structure, this study intended to investigate the potential effect of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells.

Objectives

1. To study the effects of xanthoxylin on melanin content and dendriticity of mouse B16F10 melanoma cells.
2. To investigate the mechanisms of action of xanthoxylin on
    a. the mRNA expression of regulatory proteins of melanogenesis.
    b. its signal transduction pathways

Hypothesis

Xanthoxylin stimulates the melanogenesis and dendricity in mouse B16F10 melanoma cells.

Keywords

melanogenesis / hypopigmentation / melanin / mouse B16F10 melanoma cells / Xanthoxylin

CHAPTER II

LITERATURE REVIEWS

The skin

The skin covers the entire surface of the human body. It has three layers, the epidermis, dermis, and subcutaneous layer (or fat layer). The epidermis is the external layer of the skin. It is a stratified, squamous, nonvascular, epithelium. It is composed of keratinocytes, which form different sub-layers on the surface with constantly dying and renewing, Langerhans cells which involve in the epidermal immune system, basal cells, and melanocytes which produce skin pigments called melanin [8, 22]. The dermis is an underlying connective tissue consisting fibroblasts, vascular, neural and lymphatic systems. The hypodermis or subcutaneous layer is the last layer including fatty connective tissue that connects the dermis to underlying skeletal components (Fig. 1). It skin functions as a protective barrier against several harmful environment. It involves in maintaining the proper body temperature. It gathers sensory information from the environment, and plays an active role in the immune system protecting the body from microbial [8, 22]. It also protection of the ultraviolet radiation (UVR) by absorbing pigmentation system and the complex immune regulatory networks [8].

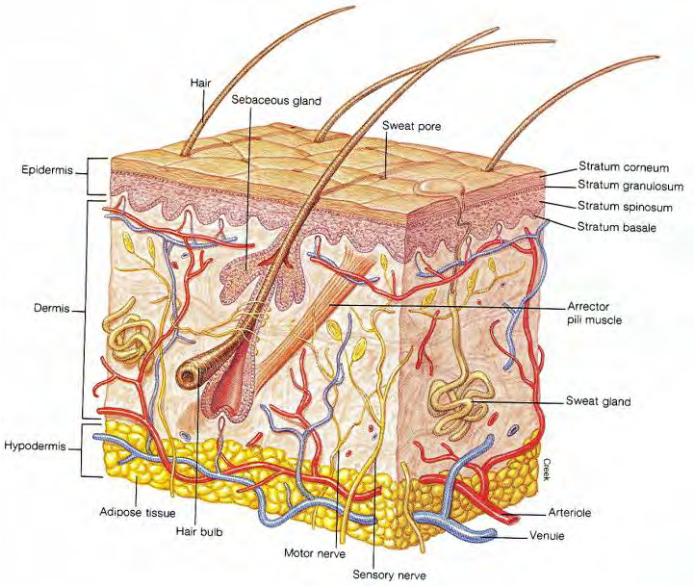


Figure 1: Human skin structure, which are three layers including epidermis, dermis and hypodermis [23].

Melanocytes, melanin, and melanogenesis

Melanocytes are melanin-producing cells derived from pluripotent neural crest cells that differentiate into numerous cell lineages, including neurons, glia, smooth muscle, cartilage, craniofacial bone and melanocytes [24]. During embriogenesis, melanoblasts migrate to various tissues including the epidermis, hair follicles, the middle layer of the eye (the uvea), the inner ear, and meninges to proliferate and differentiate into melanocytes by the influence of a number of signaling molecules produced by neighboring cells [8, 24]. These signaling molecules include Wnt, endothelin-3 (ET3), bonemorpho-genetic (BMPs), steel factor and hepatocyte growth factor (HGF). They interact with their specific cell surface receptors, induce intracellular and intranuclear signaling to influence various gene transcription and protein synthesis [24].

The crucial role of melanocyte is produce melanin pigment for photo-protective role against the carcinogenic and deleterious effects of ultraviolet radiation of solar light. Melanins are hydrophobic polymers and multifunctional biopolymers. Two different kinds of melanins are produced by melanocytes: the lighter red/yellow, alkali soluble sulfur-containing pheomelanin and eumelanin, dark brown/black insoluble pigments [10, 25]. Human skin normally contains a mixture of all two types of melanin in different ratio that varies greatly and determines the color of the skin. These pigments are synthesized and stored in cytosolic orgranelles called melanosome [2, 8, 10, 26].

For melanin production or melanogenesis, melanocytes begin to form melanosome, a specialized intracellular organelle that originates from the endoplasmic reticulum. There are four stages of melanosome maturation determined by their structure and the quality, quantity and arrangement of melanin products (Fig. 3 and Fig. 2). Newly formation melanosomes are located at the perinuclear region near the Golgi apparatus for receiving all enzymes and proteins required for melanogenesis.

Stage I melanosomes; they are spherical vacuoles lacking tyrosinase which is the key enzyme in melanogenesis and have no internal structural components. Melanosomes in stage I already contain the melanosomal proteins PMEL17/GP100

(protein required for melanosome matrix formation) and MART-1 (protein required for the function of the PMEL17/GP100 and the maturation of melanosomes) [2, 8, 24, 27].

Tyrosinase and TRP-1 are initially synthesized in the endoplasmic reticulum, become mature proteins in the Golgi apparatus and the trans-Golgi network, and packaged in endosome before fusing with melanosome in stage II by adaptor protein 3(AP-3) [2, 24]. TRP-2 is also matured in similar way as tyrosinase and TRP-1 but it is directly packaged in melanosome stage II (Fig. 2).

Stage II melanosomes; they contain mature tyrosinase, TRP-1 and TRP-2 with minimal amount of melanin. Melanosome at this stage have corrected processing PMEL17 which is an important melanosomal structural protein. PMEL 17 determines the transformation of stage I melanosomes to elongated, fibrillar stage II melanosomes [8].

Stage III melanosomes; Melanin synthesis is started and the pigment is uniformly deposited on the internal fibrils in this stage of the melanosomes [8].

Stage IV melanosomes; these melanosomes are detected in highly pigmented melanocytes. They are either elliptical or ellipsoidal, electron-opaque due to complete melanization, and have minimal TYR activity [8].

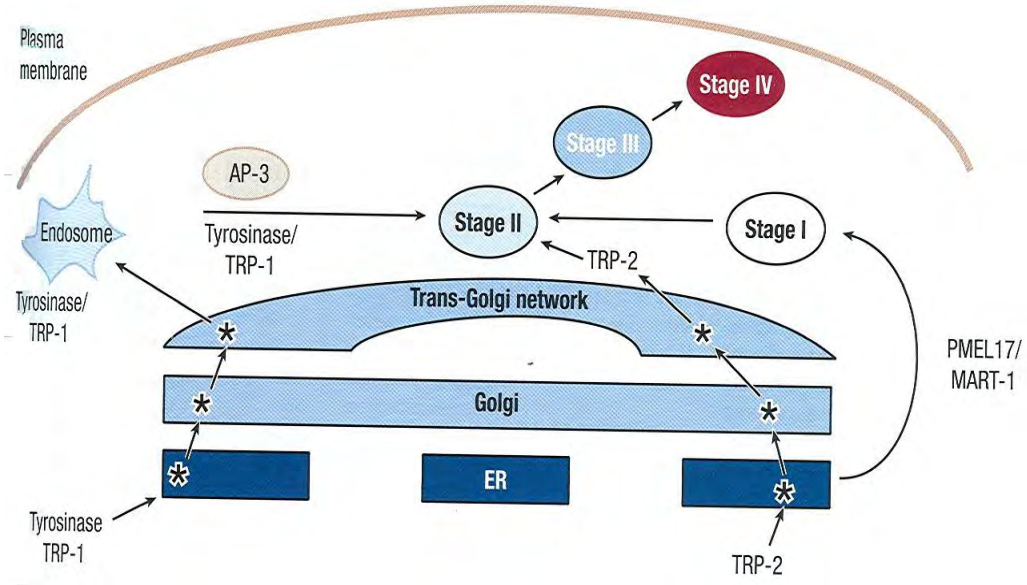


Figure 2: Sorting of melanosomal protein into melanosomes [24].

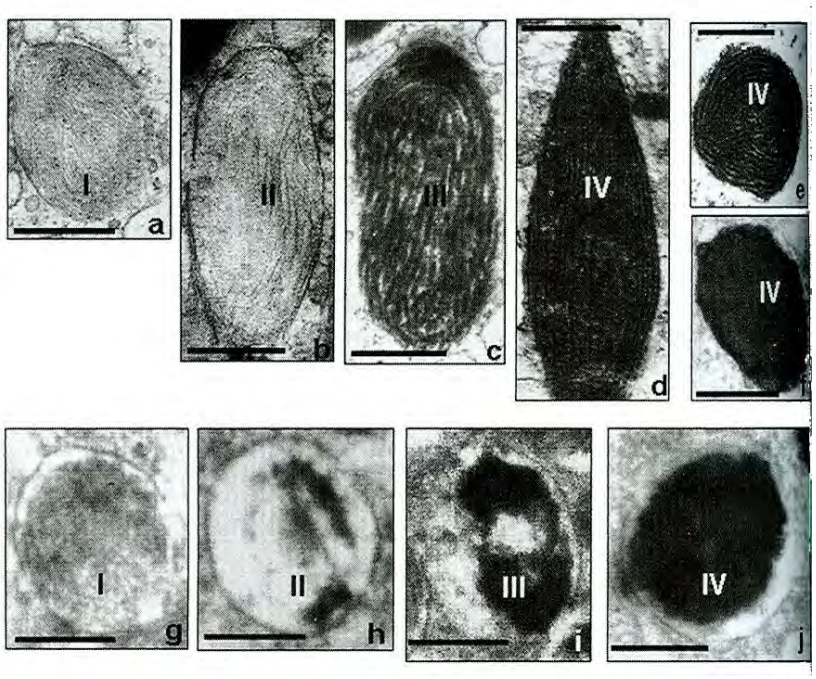


Figure 3: Electron microscopy of melanosome. Development of eumelanosome (a-f) and pheomelanosome (g-j) in stage I, II, III and IV with different maturation stages of melanosomes [24];

-In stage I melanosome develop from the endoplasmic reticulum corresponds to the early matrix organization

-In stage II, in eumelanosomes: the matrix is already organized without melanin formation contrast with pheomelanosomes which melanin is already formed

-In stage III, melanin is deposited

-In stage IV, melanin are completely full in melanosomes

Melanins are the products of multi-step biotransformation of L-tyrosine. Melanin synthesis in melanosome is initiated either directly from L-tyrosine which occurs both *in vivo* and *in vitro* or from hydroxylation of L-phenylalanine to L-tyrosine which occurs only *in vivo*. L-tyrosine is hydroxylated to L-dihydroxyphenylalanine (L-DOPA) by either tyrosinase or possibly by tyrosine hydroxylase itself. Next, L-DOPA is oxidized to dopaquinone. This oxidation is the common step for both eumelanin and pheomelanin biosynthesis. For eumelanin synthesis; dopaquinone is further transformed by a series of oxidoreduction reactions to produce two intermediates dihydroxyindole (DHI) and DHI

carboxylic acid (DHICA). These intermediates then undergo polymerization to form eumelanin. For pheomelanin synthesis; dopaquinone is conjugated to cysteine or glutathione to cysteinyldopa and glutathionyldopa which are further transformed to pheomelanin (Fig. 4). While three enzymes are absolutely required for eumelanogenesis including tyrosinase, tyrosinase-related protein 1 (TRP1) and DOPAchrome tautomerase (DCT), only tyrosinase is essentially required in pheomelanogenesis [8, 24, 27-30].

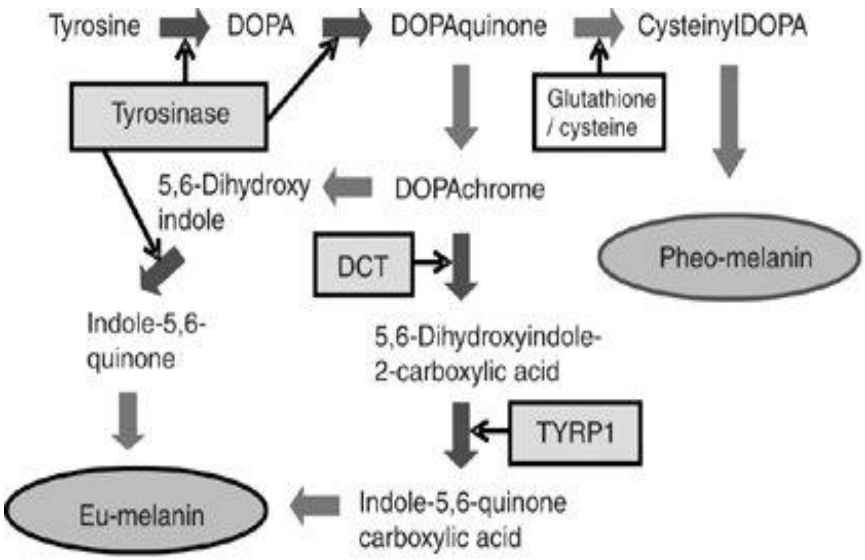


Figure 4: Melanin biosynthesis in melanocytes [30].

Important enzymes and proteins in melanogenesis

-Tyrosinase

Tyrosinase is a multifunctional, glycosylated, copper-containing monooxygenase enzyme with a molecular weight of 60-70 kDa located in the melanosomal membrane [3, 31]. It is encoded by the TYR gene on chromosome 11q14-21 in humans or chromosome 7 in mice [32, 33]. Its structure has high homology with TRP1 and TRP2 (Fig. 5). It is a single chain membrane glycoprotein which structure is highly conserved among species and has high homology with TRP1 and TRP2 (Fig. 5 and Fig. 6). The N-terminal domain of this enzyme consists of the NH_2-terminal signal peptide which is

important for intracellular trafficking and processing, two cysteine-rich domain which may serve as a protein binding/regulatory domain, and two histidine-rich regions binding copper which are the catalytic domain, while the C-terminal domain consists of hydrophobic transmembrane segment and cytoplasmic tail which are necessary for targeting the enzyme to the melanosome. The catalytic site of tyrosinase contains two copper atoms ligated to six histidine residues. Proper folding of newly synthesized tyrosinase in the endoplasmic reticulum (ER) is important for its further transport to Golgi apparatus [2].

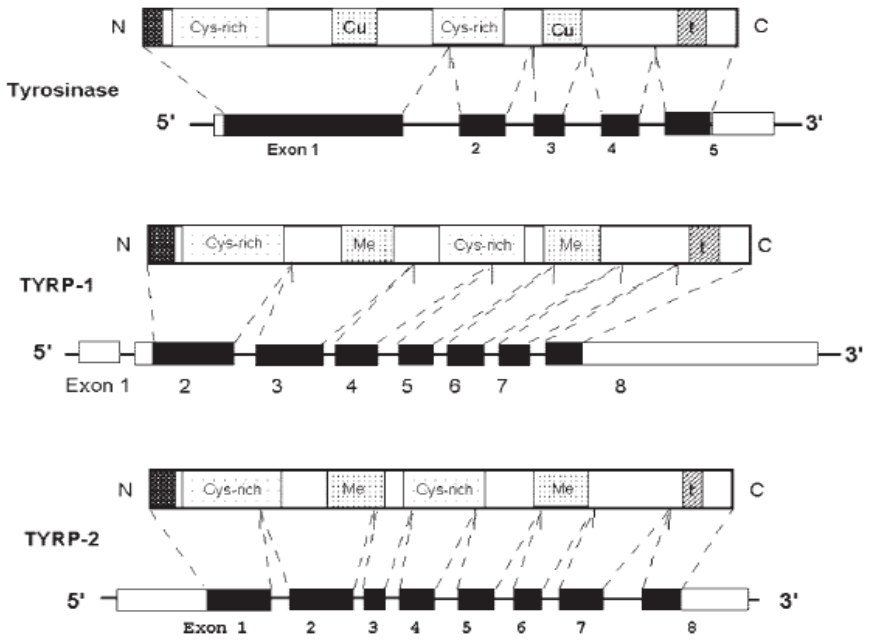


Figure 5: Gene and protein structures of tyrosinase, TRP-1 and TRP-2 with represent exons. N and C: amino and carboxy protein terminus, Cy-rich: cysteinerich segments, Cu: Cu binding domains, Me: metal binding domains, t: transmebrane segment [2].

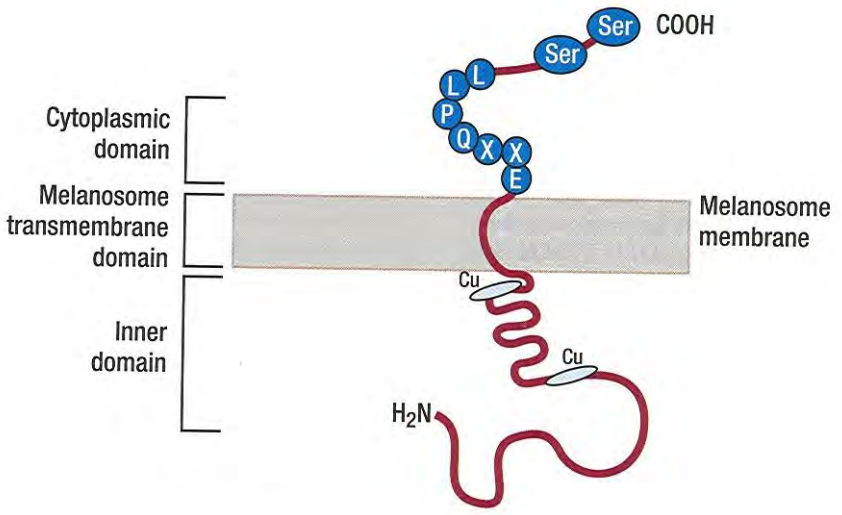


Figure 6: Structure of human tyrosinase with copper-binding sites. Human tyrosinase is a single transmembrane protein. The catalytically active domain with copper-binding site of the enzyme resides within melanosomes while a small enzymatically non-essential part of the enzyme extends into the cytoplasm of the melanocyte [24].

Tyrosinase is the key enzyme in the initial and rate-limiting reaction of melanogenesis. It catalyzes three different reactions in the melanogenic pathway. It catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), the oxidation of DOPA to DOPAquinone, and the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone [34-36].

-Tyrosinase-related proteins

Tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2) are two additional proteins involve in eumelanogenesis. TRP1 is encoded by the b-locus located on mouse chromosome 4 and TYRP1 locus on human chromosome 9, and TRP2 or DOPAchrome tautomerase is encoded by the slaty locus on mouse chromosome 14 and TYRP2 on human chromosome 13 [2, 24, 37]. TYRP1 and TYRP2/DCT proteins have structures similar to and share about 40% amino acid homology with tyrosinase (Fig. 5). Both TRP1 and TRP2 contain an NH_2-terminal signal sequence, EGF-like domains, and

other cysteine-rich region, two histidine-rich metal binding domains, and a COOH-terminal transmembrane segment with short cytoplasmic tail.

TRP-2 has two highly conserved zinc-binding sites with three histidines on each side that bind to zinc. It acts as DOPAchrome tautomerase by catalyzing L-dopachrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) while TRP-1 contains iron-binding site in histidine-rich metal binding domain. It acts as DHICA oxidase by catalyzing the conversion of 5,6-dihydroxyindole-2-carboxylic acid into eumelanin [38, 39]. TRP-2 and TRP-1 are also involved in other cellular functions, like cell viability [40], growth and morphology [41], cell-cell contacts [42] or maturation and stability of melanosomes [43].

Melanogenic regulatory proteins

-Microphthalmia-associated transcription factor (MITF)

MITF is a key transcription factor in melanin biosynthesis. It is a transcription factor of the basic-helix–loop–helix-leucine-zipper family (bHLHzip) [5, 35]. It is encoded by the *MITF* gene on chromosome 6 in mouse and chromosome 3p12.3–14.1 in humans [45]. There are at least five isoforms of MITF arising from multiple alternative promoter and initial exon usage. The M isoform of MITF is specifically expressed in melanocytes. The amino terminus in the MITF-M isoform is encoded by the melanocyte-specific exon 1 and its expression is exclusive to the melanocyte lineage because of its unique melanocyte restricted promoter enhancer.

MITF positively regulates transcription of genes that encode to key enzymes in melanogenesis including tyrosinase, TRP1 and TRP2 [46-49]. These enzymes are transcriptionally regulated by a tissue-restricted cis-acting promoter containing a canonical E-box or M-box. Phosphorylated MITF binds to either M-box or E-box consensus sequences to activate transcription of these proteins [46]. MITF also regulates proliferation, and survival of melanocytes [47].

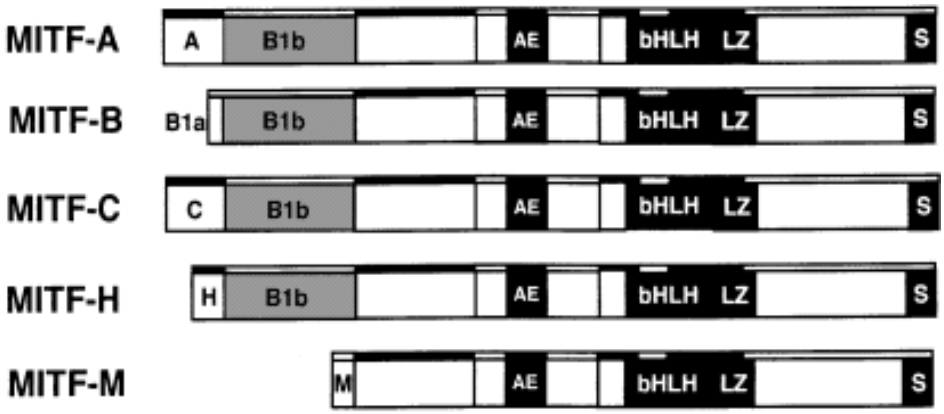


Figure 7: Structures of the MITF isoforms which share the entire carboxy part but differ at their N-termini. The transcriptional activation domain (AE) and the serine-rich region (Ser) are indicated [50].

Signaling pathways regulating melanogenesis of melanocytes

There are several signaling pathways that modulate melanin synthesis in human melanocyte. The main signaling pathway of the melanocortin system is cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway. The inositol phosphate/protein kinase C (IP3/PKC) and nitric oxide/protein kinase G (NO/PKG) pathways also can modulate the melanogenesis [2, 51]. Recently, the p38 mitogen-activated protein kinase (MAPK) cascade is also reported to involve in the induction of melanogenesis in B16 mouse melanoma cells by α-MSH [52].

-Cyclic Adenosine Monophosphate (cAMP) pathway

The cAMP-mediated pathway is a well-known signaling cascade and the most important pathway of melanogenesis regulation. The cAMP/protein kinase A (PKA) cascade is triggered by stimuli (ACTH or α-MSH) which bind to the melanocortin 1 receptors (MC1R) which is G protein ($G_{\alpha s}$)-coupled heptahelical receptor. This leads to activate adenylate cyclase (AC), generate the rise of intracellular cAMP concentration, activates PKA [53, 54]. Next, PKA activates responsible element binding protein (CREB) by phosphorylation. Phosphorylated CREB interacts with CREB binding protein (CBP)

become the complex that binds to the cAMP response element (CRE) motif of the MITF promoter and induces the expression of MITF. Finally, MITF acts as positively regulator to increase the expression of tyrosinase, TRP-1 and TRP-2 enzymes (Fig. 8) [55, 56-58].

In addition, cAMP associates to other signaling pathways involve in melanin synthesis. It inhibits AKT activation through phosphatidylinositol 3-kinase (PI3K)-dependent mechanism by inhibited the phosphorylation of AKT at threonine 308 and serine 473. This inhibition leads to activation of glycogen synthase kinase-3 β (GSK-3β) by dephosphorylation. Dephosphorylated GSK-3β is the active form of the enzyme that phosphorylates MITF on serine 298 to enhance this transcription factor binding to the tyrosinase promoter (Fig. 8) [59-62].

However, cAMP-stimulating agents can cause the decrease in melanogenesis through the activation of the Ras/MEK/ERK pathway. cAMP activates the Ras, B-Raf, MEKK, ERK, and Rsk-1 cascade. ERK and Rsk-1 phosphorylate MITF on serine 73 and serine 409, respectively (Fig. 8). This phosphorylated MITF promotes its degradation and leads to the inhibition of melanogenesis. This pathway has been thought to be a feedback mechanism of melanogenesis to prevent an excessive production of melanin that would be toxic to melanocytes [61, 63].

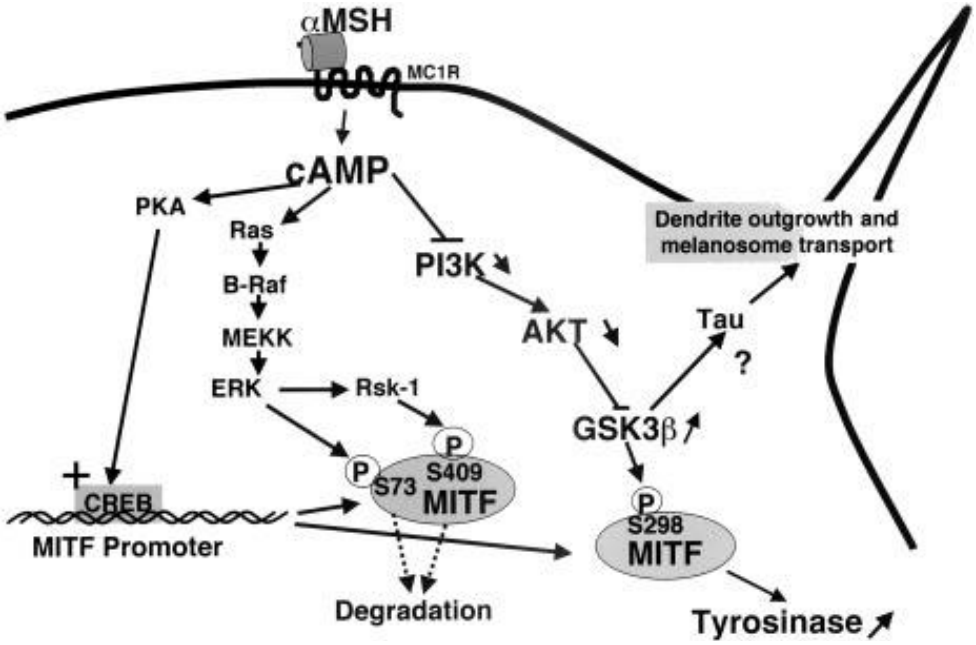


Figure 8: Signaling pathways involved in cAMP-induced melanogenesis [61].

-Protein kinase C (PKC) pathway

PKC is a serine/threonine kinase family that mediates various cell functions, such as cell proliferation and differentiation. It is in inactive form in the cytoplasm and activated by diacylglycerol (DAG) when cell surface receptors interact with ligands [24]. There are at least 12 isoforms of PKC. It has been hypothesized that specific intracellular localization of a PKC isoform leads to its specific biological function. Human melanocytes express $\alpha, \beta, \delta, \varepsilon$ and ζ isoforms of PKC but only PKC-β is shown to be associated with melanosome in these cells [3, 64]. Normally, inactive PKC-β is in the cytoplasm of melanocytes but activated PKC-β is associated with the melanosomal membrane. Some stimuli of melanogenesis, such as endothelin-1 (ET-1), activate phospholipase C, lead to the release of diacylglycerol (DAG) which can activate PKC-β. Activated PKC-β binds the Receptor for Activated C-Kinase-I (RACK-I) which is the protein functions in stabilization the active form of PKC-β and translocates this enzyme to melamosomal membrane to phosphorylates tyrosinase (Fig. 9) [3, 24, 65].

PKC-β activates tyrosinase by phosphorylating the enzyme specifically at serine 505 and 509 in the cytoplasmic domain [59]. Phosphorylated tyrosinase forms complex with TRP-1 which leads to stabilization of tyrosinase and increase its enzymatic activity [65].

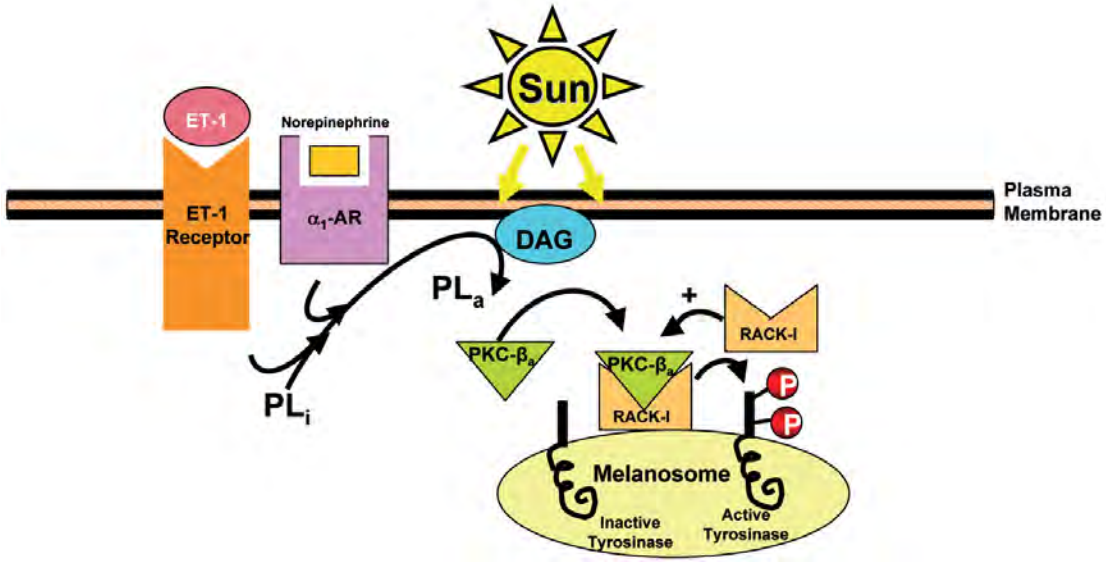


Figure 9: Activation of tyrosinase by PKC-β. UV irradiation, ET-1 and norepinephrine binding with their specific receptor cause releases DAG from the cell membrane and activated PKC-β by binding RACK-I and then phosphorylates serine residues on tyrosinase [3].

- p38 mitogen-activated protein kinase (MAPK) pathway

It has been revealed that p38 MAPK cascade was also involved in melanogenesis in B16 cells induced by cAMP-elevating agents α-MSH, human placental lipid (PTLF) and UV light or Lupeol [66-68]. PTLF and UV-mediated phosphorylation of p38 MAPK cascade resulted in phosphorylation of transcription factors such as MITF and USF-1 that regulate the transcription of proteins involve in melanogenesis especially tyrosinase and TRP1 (Fig. 10) [67, 69-71].

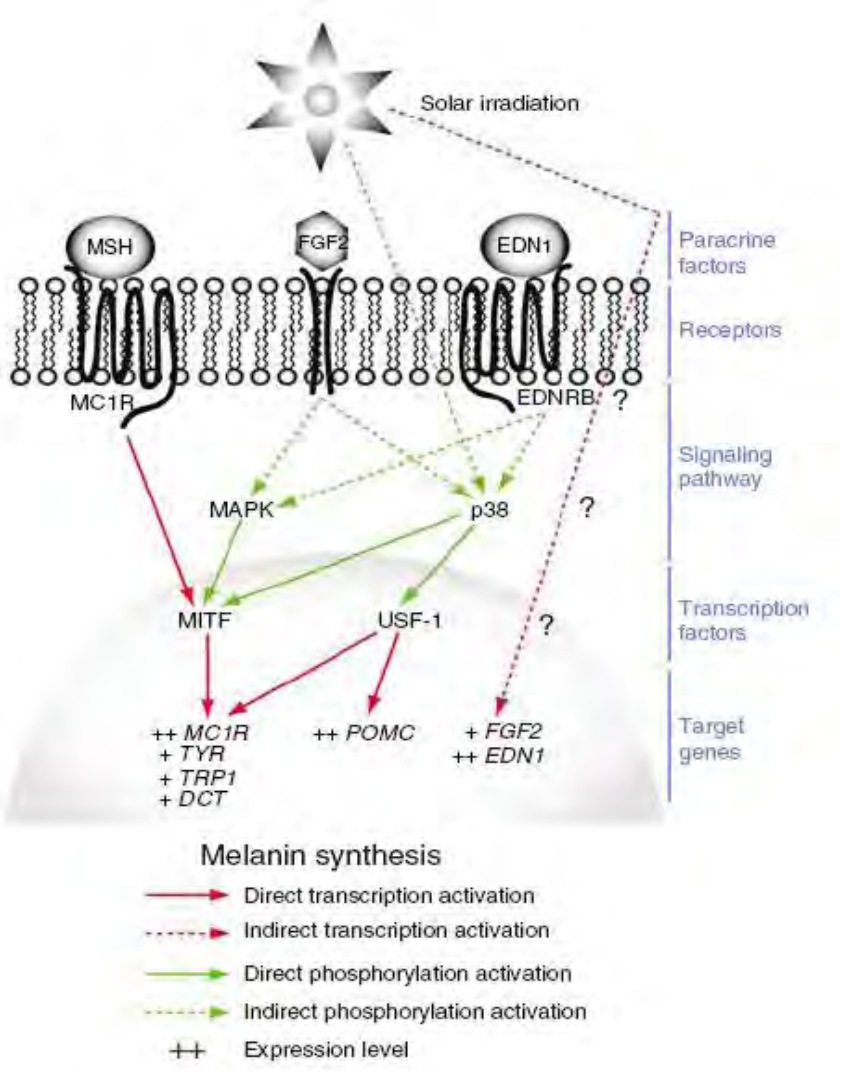


Figure 10: Signaling of UV-induced pigmentation. UV exposure results in the stress-specific p38 MAPK pathway is activated which direct phosphorylation of the USF-1 transcription factor leads to induction of MITF expression and finally increase melanin production [71].

-Cyclic Guanosine Monophosphate (cGMP) pathway

Nitric oxide (NO) has been demonstrated to stimulate melanogenesis by using UV-exposed melanocytes co-cultured with keratinocytes or NO donor. It increased in both tyrosinase activity and protein levels. The cyclic GMP is demonstrated to be a primary pathway in NO-stimulated melanogenesis. It has been postulated that cGMP activate PKG which phosphoryltes AP-1 transcription factor to induce tyrosinase

expression in melanocytes [24, 72, 73]. Additionally, It was reported that cGMP inhibited cAMP phosphodiesterase and led to an increase of cellular cAMP contents (Fig. 11) [74].

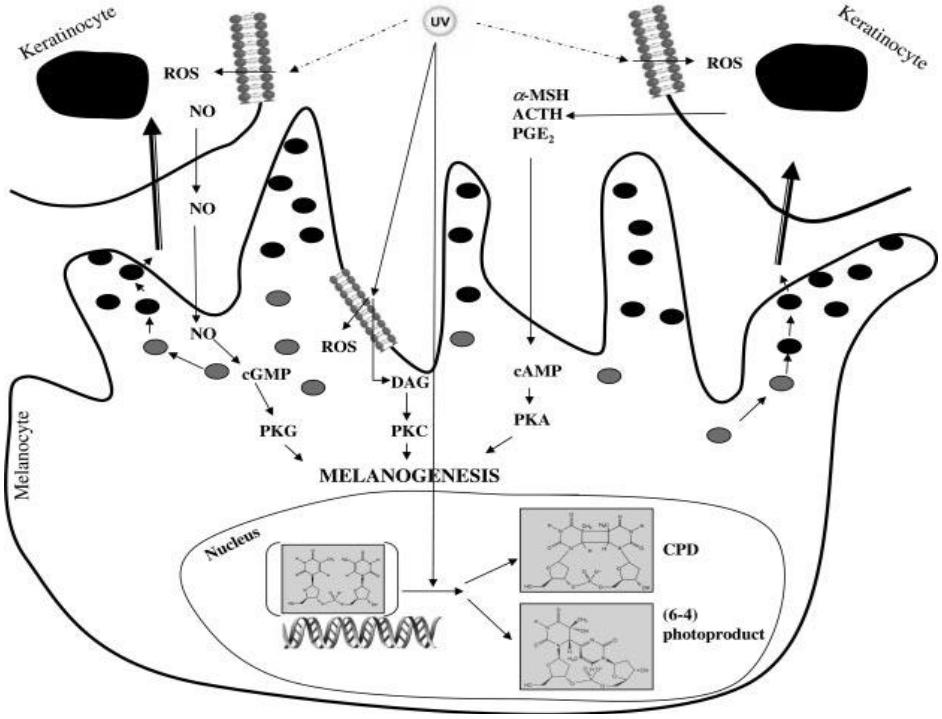


Figure 11: Signaling of UV-induced pigmentation involves NO/cGMP pathway [8].

Stimulation of melanogenesis

Many stimuli can induce melanogenesis of melanocytes. They are from several sources including neighboring keratinocytes, autocrine signals, and environmental factors. Some stimuli such as UV irradiation directly stimulate melanocyte or indirectly effects on keratinocytes to secrete mediators which stimulate melanocytes to produce melanin and stimulate dendricity of melanocytes for transferring melanosomes to keratinocytes [1, 24, 75].

-cAMP elevating agents

-α-Melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH). These hormones are potent stimulators of melanogenesis. Following exposure to UV radiation, melanocytes as well as keratinocytes express higher amounts of pro-opiomelanocortin (POMC) which is the precursor of the melanocortins, α-MSH and ACTH. These hormones bind to the melanocyte melanocortin receptor type 1 (MC1R) and stimulate intracellular signaling responses in melanocytes though cAMP system. The increase in the cAMP content in melanocytes leads to increase the expression of melanin-producing enzymes [52, 70, 72, 76].

-Forskolin. Forskolin is a cell permeable diterpenoid from root of *Coleus forskohlii* [10]. It is a well known α-MSH agonist or exogenous cAMP elevating agent [77] that activates adenylate cyclase which increase cAMP content leading to increase protein levels of MITF, tyrosinase, TRP-1 and TRP-2 [78, 79].

-Isobutylmethylxanthine (IBMX). IBMX is known to increase the cellular cAMP through the inhibition of the cAMP-degrading enzyme, phosphodiesterase . It increases tyrosinase activity, protein and mRNA expression leading to increase melanogenesis [79, 80].

-Glycyrrhizin. Glycyrrhizin is natural product from roots and rhizomes of licorice, *Glycyrrhiza glabra* L. It induces melanogenesis through cAMP signaling pathway. It activates PKA to phosphorylate CRE binding protein (CREB) transcription factor that induces the expression of MITF transcription factor. Glycyrrhizin also inhibits GSK-3β phosphorylation. Dephosphorylated GSK-3β restores its kinase activity to phosphorylate MITF. This leads to enhance the binding of MITF to the tyrosine gene promoter [59].

-Protein kinase C activators

-1-Oleoyl-2-acetylglycerol (OAG). OAG is an analogue of diacylglycerol. It stimulates PKC which leads to tyrosinase phosphorylation and activation of melanogenesis [81].

-Endothelins-1 (ET-1). ET-1 stimulates melanogenesis by binding to specific receptor of ET-1 on melanocytes. It increases phosphatidylinositide turnover and PKC activation which cause of activation of tyrosinase and increase TRP-1 levels [82]. It also stimulates proliferation of melanocytes and promotes dendrite formation [83-85]. Moreover, ET-1 upregulates the MC1R level and increases MC1R affinity for α-MSH [24, 86].

-p38 mitogen-activated protein kinase (MAPK)

-Placental total lipid fraction (PTLF). PTLF containing sphingolipids induces melanogenesis through the p38 MAPK signaling cascade. It up regulates tyrosinase gene expression, increases level of tyrosinase protein and tyrosinase enzyme activity [69].

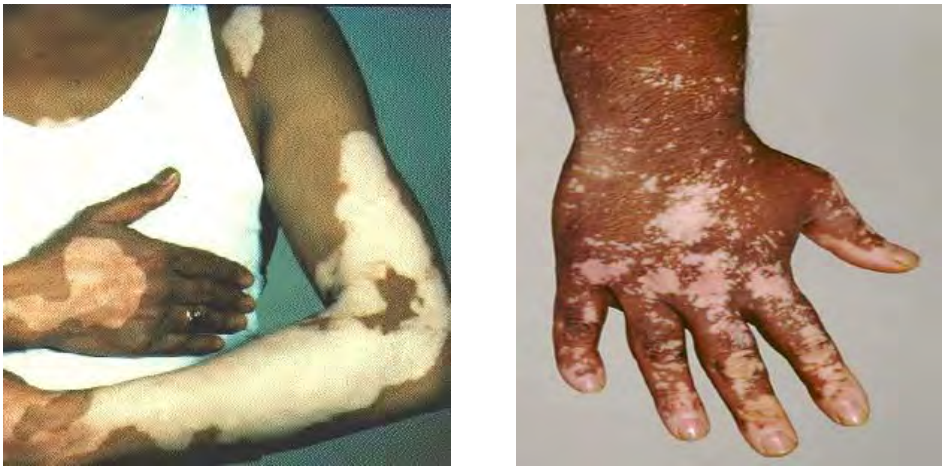
UV radiation

UV radiation is one of the most powerful stimulator of melanogenesis. It causes direct effects on melanocytes to increase melanogenesis. It activates phospholipase C to modify membrane phospholipids by causing the generation of diacylglycerol (DAG). DAG activates PKC which phosphorylates tyrosinase and turns the enzyme to active form. UV radiation also acts indirectly through keratinocytes to stimulate melanogenesis in melanocytes. It induces the release of several factors from keratinocytes, such as α-MSH, ACTH, ET-1 and NO, to stimulate melanogenesis [76, 87, 88]. Additionally, UV also activates through p38 MAPK pathway to phosphorylate the MITF or and USF-1 transcription factors that involve in melanogenesis [69].

Hypopigmentation

Hypopigmentation, hypomelanosis, or skin depigmentation is a decrease in normal skin color resulting mainly from two types of changes; the decrease in number or the absence of melanocytes which results in reducing melanin synthesis such as vitiligo and the decrease in melanin production without changing in the number of melanocytes [89]. It may be congenital or acquired sign. Hypopigmentation can be resulted from genetic disorders, autoimmune disorders, chemicals and drugs, inflammation, infection, and physical trauma. Contact with certain melanotoxic chemicals can lead to skin depigmentation. The majority of these agents are phenol and catechol derivatives. They can cause hypopigmentation at the contact sites (leukoderma). Chronic exposure of these agents can lead to vitiligo. Skin depigmentation can occur post-inflammation, such as after resolving of psoriatic plaques or lesions of atopic dermatitis. This disorder is possibly resulted from an increased keratinocyte turnover which decreases melanosomal transfer or the activation of inhibitory cytokines. Infection can also cause hypopigmentation. *Mycobacterium leprae* contains an enzyme similar to TYR that potentially converts DOPA to a quinone, so that DOPA is unavailable for melanin production [76]. Vitiligo is usually slow progressive skin depigmentation with different clinical patterns. Hypopigmentation in vitiligo is caused by the loss of melanocytes. Several different theories have been implicated in the pathogenesis of the disease: the autoimmune, the neuronal, the self-destructive, and the genetic (Fig. 12 and Fig. 13) [89, 90-92].

Many physical agents including heat, freezing, x-ray, ionizing radiation, UV irradiation and laser light can cause hypopigmentation or permanent depigmentation by damaging melanocytes [24].



(A) (B)

Figure 12: Vitiligo. (A): Vitiligo with very extensive lesions but clear demarcation, (B): Vitiligo of the hand with very obvious on dark skin [93].

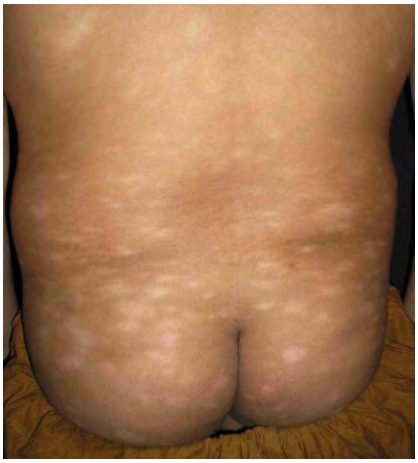


Figure 13: Typical lesions of hypopigmented mycosis fungoides seen over the buttocks and trunk [94].

Skin pigment enhancers

Melanin in the epidermis provides protection against DNA damage and skin cancer caused by UV radiation from sunlight. Agents that increase melanin synthesis have the potential to reduce photodamage and skin cancer incidence. They also are potentially useful to treat some types of hypopigmentation such as vitiligo. The choices

of vitiligo therapy depends on the age of the patient, the clinical pattern of the disease (type, severity, activity) the psychologic impact and the risk-to-benefit ratio of prolonged therapy [92, 95]. Many agents have potential to use as skin enhancers but very few are clinically used.

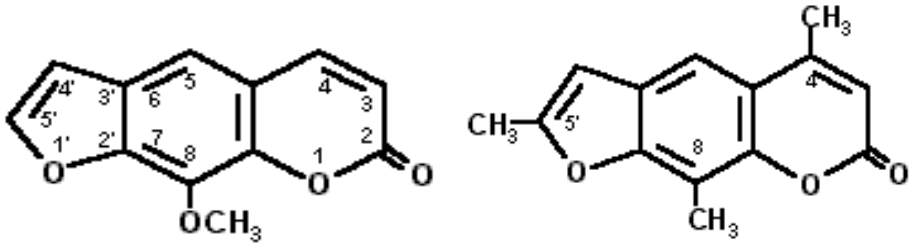
Photochemotherapy or psoralen ultraviolet A (PUVA) therapy

PUVA is a combination of psoralens and long wave UV radiation; UVA used to induce melanogenesis. UVA is mainly responsible for indirect DNA damage by the generation of reactive oxygen species (ROS) and result in single-strand breaks in DNA and in DNA-protein crosslinks [96]. Absorption of photons by psoralen in the UVA range results in the formation of a 3,4- or 4',5'-cyclobutane addition product with pyrimidine base of native DNA. In cultured human melanocytes, the cyclobutane adducts increase activity of PKC that is important in melanogenesis [14, 97-98].

Skin pigmentation can be induced either by injection, oral ingestion, or by topical application of psoralens prior to UVA exposure. Topical and systemic psoralen-ultraviolet A (PUVA) therapy, are at present the most effective treatments available to induce repigmentation of vitiligo lesions [15, 99].

In addition, psoralens can directly stimulate melanogenesis by increasing the activity and synthesis of tyrosinase in the absence of UV [100].

Psoralens, such as 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP)/bergapten, are natural tricyclic furocoumarin present in many plants (Fig. 14). 4, 5', 8-Trimethylpsoralen (TMP) is a synthetic compound is also used (Fig. 14) [14].



(A) **(B)**

Figure 14: Molecular structure of (A): 8-MOP and (B): TMP [101]

8-MOP can directly stimulate the production tyrosinase by increasing the expression of MITF transcription factor and by subsequently increasing the stability and enzymatic function of tyrosinase though PKA signal pathway [15].

The side effects of photochemotherapy such as nausea, phototoxic reactions, and long-term carcinogenic risk, are of great concern [102].

-L-Tyrosine

L-Tyrosine was developed for melanogenesis by the thought to induce melanin synthesis by increasing substrate of melanogenic pathway. It was found that L-Tyrosine increased the melanin content in melanoma cell much higher than in normal human melanocyte but lack of response in animal skin [13].

-L-Dihydroxyphenylalanine (L-Dopa) and L-Dopa phosphates

L-Dopa acts as the product, the substrate as well as the essential cofactor of tyrosinase in the initial steps of melanin synthesis. It was used in L-DAPA phosphate due to its water insolubility and it is rapidly oxidized by oxidizing agents. L-DOPA phosphate is water soluble and stable in the presence of oxidizing agents. It increased the effect of α-MSH and UV radiation to induce melanin synthesis. However the major concern of L-DOPA and L-DOPA phosphate is it's cytotoxic on melanocytes while during melanogenesis melanocytes are protected from this toxicity by the partitioned of L-DOPA in melanosomes [13].

-Diacylglycerols (DAGs)

Activation of DAG and stimulation PKC signaling pathway by UVA in melanocytes leads to phosphorylation of tyrosinase and activation tyrosinase activity for melanogenesis. It has been studied that 1-oleyl 2-acetyl glycerol (OAG), a synthetic DAG, increased melanin content in both melanoma cells and normal melanocytes [103]. It also acted synergistically with UV radiation in cultured cells as well as animal skin [104]. OAG and other DAGs appear to have potential to use as skin enhancers to

induce melanin synthesis. However safety issues of these agents are the major concern. Topical application of DAGs may cause local inflammation [82]. Activation of PKC by UVA has been associated with tumor promotion both in animal and in cell culture [105].

-Melanocyte stimulating hormone (MSH) and peptide analogs

MSH and its analog [Nle^4-D-Phe^7]-α- MSH can induce pigmentation via cAMP signaling pathway. It has been reported that topically applied or subcutaneously injected MSH and this analog induce pigmentation in animal skin [13]. They also increased tyrosinase activity and melanin content in S91 melanoma cells [106]. Subcutaneously injection MSH analogs [Nle^4-D-Phe^7]-α- MSH and Melanotan-II for 10 days increased skin pigmentation in human [107, 108]. Potent tetrapeptide α-MSH analogs, *n*-Pentadecanoyl- and 4-Phenylbutyryl-His-D-Phe-Arg-Trp-NH_2, were able to stimulate melanogenesis and enhance DNA repair after UVR of melanocytes *in vitro* [109].

Side effects of MSH analog injections include erythematous flushing, yawning, lethargy, nausea and spontaneous penile erections [107, 110]. These side effects, the unevenness of tanning and the inconvenience of administration by subcutaneously injection are major reasons to limit the use of MSH analogs as skin enhancers.

-3-Isobutyl-1-methylxanthine (IBMX)

IBMX is a well known and potent activator of melanin synthesis. It is a phosphodiesterase inhibitor. It is suggested that IBMX stimulates melanogenesis by inhibiting phophodiesterase which inactivates cAMP, so increasing levels of cAMP. IBMX is found to increase tyrosinase activity and melanin synthesis in cultured cells but no evidence of efficacy in animal or human skin. IBMX has been demonstrated to both promote and inhibit carcinogenesis [13].

-Bicyclic monoterpene (BMT) diols

The discovery of these diols induce melaninogenesis was originated from the observation that propylene glycol (1, 2-propanediol), a vehicle of topical melanogenic agents also induced melanogenesis itself. BMT diols are found abundantly in plants and food. They have been demonstrated to result in increase tyrosinase activity and melanin content, both in cultured cells and animals. They stimulate melanogenesis in similar mechanism to UV radiation by inducing NO production and cGMP activation [77]. Further studies are needed to determine any side effects of BMT diol resulted from activation of NO production.

Other agents that have been demonstrated to increase tyrosinase activity and melanin synthesis in cultured cells without evidence of effectiveness in animals and humans are lysosomotropic agents, such as ammonium chloride, which increase the melanosomal pH, NO donors which can activate cGMP, and small DNA fragment thymidine dinucleotide (pTpT) which are generated during the DNA repair process following UV radiation [13].

Xanthoxylin

Xanthoxylin is a phenolic compound belongs to acetophenone present as principal compound in many plants of Rutaceae and Euphorbiaceae family [21]. It has molecular formula as $C_{10}H_{12}O_4$ and its molecular weight is 196.20 g/mol. The chemical names of xanthoxylin are 2,4-dimethoxy-6-hydroxyacetophenone and 2'-hydroxy-4',6'-dimethoxyacetophenone (Fig. 15). The other synonyms of xanthoxylin are xanthoxyline and brevifolin. It is colorless plates with melting point (mp) 81-83°, practically insoluble in water and soluble in alcohol and ether. Xanthoxylin isolated from *Zanthoxylum americanum* have been used as stimulant for chronic rheumatism, typhoid, skin disease and impurity of the blood [111].

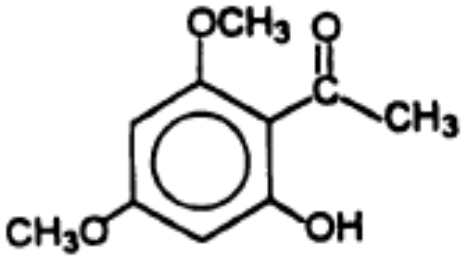


Figure 15: Molecular structure of xanthoxylin [17].

Pharmacological effects of xanthoxylin

Some pharmacological activities of xanthoxylin has been evaluated.

-Antinociceptive and antioedematogenic activities

It has been studied that a derivative of xanthoxylin, (2-(4-bromobenzoyl)-3-methyl-4,6-dimethoxy benzofuran) or BMDB ($C_{17}H_{15}O_4Br$) (Fig. 16) exhibited dose-dependent spinal and supraspinal antinociception in mice administered by i.p., s.c., subplantarly or i.c.v. route. Its antinociceptive action was determined in several analgesic models including the neurogenic nociception responses induced by formalin and capsaicin [17].

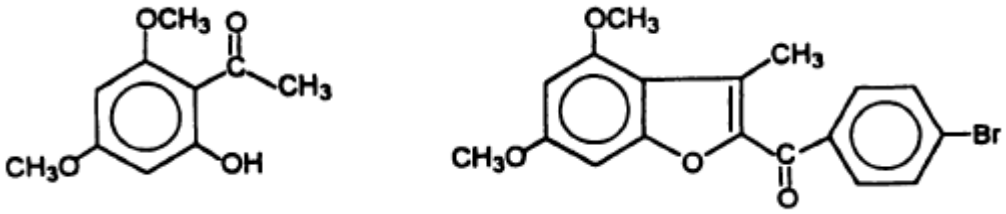


Figure 16: Structure of xanthoxylin (A) and BMDB which an xanthoxylin derivative (B) [17].

Xanthoxylin administered by i.p. showed partial antinociceptive effect in acetic acid-induced abdominal constriction in mice while some xanthoxylin derivatives exhibited more potent analgesic effect in the same study. Xanthoxylin and its derivatives also inhibited the formation of paw oedema caused by dextran and strongly prevented bradykininin induced oedema formation [20].

-Antifungal and antibacterial activities

Xanthoxylin has moderate antifungal activity against several pathogenic fungi (*C. albicans* FCF-243, *C. albicans* ICB-12, *M. canis* LM-003, *M. canis* 72T, *T. rubrum* 45T, *A. parasiticus*, *A. flavus* and *Penicillium)* of xanthoxylin has been demonstrated [17, 18]. It also exhibit antibacterial activity against *Escherichia coli*, *Enterobacter cloaceae*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae* and *Morganella morganii* [112, 113].

-Antispasmodic activity

Xanthoxylin and certain derivatives, 2,3-dimethoxyacetophenone and 2,4-dihydroxy-6-methoxyacetophenon, exhibit antispasmodic effect against acetylcholine-induced contraction of guinea pig isolated ileum [19, 114].

It also has been studied that xanthoxylin inhibits prostaglandin synthetase, 5-lipoxygenase and the viability of Ehrlich ascites tumour cells [21].

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Test compounds

Xanthoxylin was purified by Assoc. Prof. Dr. Nijsiri Ruangrungsi, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. It is dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C as the stock solution. Its working solutions were prepared by diluting the stock solution with sterile double distilled water to 2% DMSO solutions.

1.2 Melanocyte cells

The mouse melanoma cells, B16F10, from the American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin in a humidified atmosphere containing 5% CO_2 in air at 37 °C. The cells were subcultured or collected for doing experiments by trypsinizaion with 0.25% (v/v) trypsin-EDTA solution.

1.3 Equipments and Instruments

The following instruments and equipments were used in this study; autoclave (Hirayama, Japan), biohazard lamina-flow hood (Science, Germany), Incubator (Thermo, USA), Light microscope (Nikon, Japan), refrigerator 4°C and -20°C (Sanyo, Japan), microplate reader (Labsystems Multiskan MS), pipettes (Falcon, USA), pH meter (Mettler tuledo, Switzerland), autopipette (Gilson, France), centrifuge (Eppendorf, Germany), gel documentation (Bio-Rad, USA), PCR thermal cycler (Eppendorf, Germany), gel electrophoresis (Bio-Rad, USA), T25 tissue culture flasks (Corning, USA), 12-well plates (Costar, USA), 96-well plate (Falcon, USA).

1.4 Reagents

The following reagents and reagent kits were used to elicit data; Dulbecco's modified Eagle's medium (Gibco, USA), fetal bovine serum (Gibco, USA), penicillin/streptomycin (Gibco, USA), sodium hydroxide, Taq DNA polymerase (Invitrogen, USA), Improme-II™ reverse transcription system (Promega, USA), agarose (Bio-Rad, USA), dNTP mix (Vivantis, Malaysia), absolute ethanol (Merck, Germany), TRIzol® Reagent (Invitrogen, USA), diethyl pyrocarbonate (DEPC) (Molekula, UK), and reagents from Sigma Chemical Co., USA which were 0.4% trypan blue dye, 0.25% (v/v) trypsin-EDTA solution, resazulin sodium salt, α-melanocyte-stimulating hormone (melanogenesis stimulator), H-89 (protein kinase A inhibitor), Ro-32-0432 (PKC inhibitor), LY294002 (phosphatidylinositol-3-kinase) inhibitor) and PD98059 (MEK1 inhibitor).

2. Methods

2.1 Melanin content assay

The assay was performed in duplication of three independent experiments (n=3) according to the following procedures;

1. Incubated B16F10 cells at the density of 1×10^4 cells/well in 12-well plates at 37 °C for 24 hours.

2. Treated the cells with 3.12, 6.25, 12.5, 25 and 50 µg/ml xanthoxylin at 37 °C for 72 hours. 0.2% DMSO solution and α-MSH were used as negative and positive control, respectively.

3. Collected the treated cells by trypsinizaion with 0.25% (v/v) trypsin-EDTA solution and centrifugation at 12,000 g for 10 minutes.

4. Removed the supernatant and add 2M NaOH solution to make the density of the cells at 8×10^4 cells/200 µl.

5. Dissolved melanin by stirring at 60 °C for 5 minutes.

6. Measured melanin content with a microplate reader at 405 nm.

2.2 Determination of cell viability

The effect of xanthoxylin on the viability of B16F10 cells was determined by resazurin assay. Resazurin dye is reduced to the deoxygenated product, resorufin, only in viable cells. The assay was performed in duplication of four independent experiments (n=4) according to the following procedures;

1. Incubated B16F10 cells at the density of 1×10^3 cells/well in 96-well plates at 37 °C for 24 hours.

2. Treated the cells with 3.12, 6.25, 12.5, 25 and 50 µg/ml xanthoxylin at 37 °C for 72 hours. 0.2% DMSO solution and α-MSH were used as negative and positive control, respectively.

3. Added 5 µl of 1mg/ml resazurin solution and incubate for 4 hours.

4. Measured the absorbance at 570 nm and 600 nm by using a microplate reader.

5. Determined cell viability by the following equation

$$\% \text{ Viability} = \frac{OD_{control}-OD_{treated}}{OD_{control}} \times 100$$

2.3 Determination of mechanisms of action of xanthoxylin on melanogenesis

Effect of xanthoxylin on signaling pathways of melanogenesis in B16F10 cells were elucidated by using the following kinase inhibitors; protein kinase A (PKA) inhibitor (H89), PKC inhibitor (Ro-32-0432), phosphatidylinositol-3-kinase (PI3K) inhibitor (LY294002) and MEK1 inhibitor (PD98059). The assay was performed in duplication of three independent experiments (n=3) according to the following procedures;

1. Incubated B16F10 cells at the density of 1×10^4 cells/well in 12-well plates at 37 °C for 24 hours.

2. Pre-treated the cells with 5 µM of H-89 (PKA inhibitor), Ro-32-0432 (PKC inhibitor), LY294002 (phosphatidylinositol-3-kinase (PI3K) inhibitor) or PD98059 (MEK1 inhibitor) at 37 °C for 1 hour and then treat the cells with 25 µg/ml xanthoxylin at 37 °C for 72 hours.

3. Collected the treated cells by trypsinizaion with 0.25% (v/v) trypsin-EDTA solution and centrifugation at 12,000 g for 10 minutes.

4. Removed the supernatant and add 2 M NaOH solution to make the density of the cells at 8×10^4 cells/200 µl.

5. Dissolved melanin by stirring at 60 °C for 5 minutes.

6. Measured melanin content with a microplate reader at 405 nm.

2.4 Measurement of mRNA expression of proteins involve in melanogenesis

The effect of xanthoxylin on the mRNA expression of tyrosinase, TRP-1, TRP-2 and MITF in B16F10 cells was determined as in the following procedures;

1. Pre-treated B16F10 cells at the density of $1x10^4$ cells/well in 12-well plates with 5µM protein kinase inhibitors (H-89, Ro-32-0432, LY294002, and PD98059) or 0.2% DMSO solution at 37°C for 1 hour.

2. Treated the cells with xanthoxylin at 37°C for 72 hours.

3. Extraction of total RNA of treated cells, cDNA synthesis and PCR product amplification as in the following procedures;

2.4.1 Preparation of total RNA

In this experiment, mouse B16F10 melanoma cells were treated with and without H-89 (protein kinase A (PKA) inhibitor) at the final concentration of 5 µM in sterile water, Ro-32-0432 (PKC inhibitor), LY294002 (phosphatidylinositol-3-kinase (PI3K) inhibitor) and PD98059 (MEK1 inhibitor) at the final concentration of 5µM in 0.2% DMSO for 1 hour. After that cells were treated with xanthoxylin. The assay was performed in duplication according to the following procedures (n=3).

1. Incubated mouse B16F10 melanoma cells in DMEM containing 10% (v/v) fetal bovine serum at the density of $1x10^4$ cells per well in 12-well plates with sample under each condition at 37°C in a humidified atmosphere of 5% CO_2 for 72 hours.

2. Removed the supernatant, lysed and homogenized in 1 ml of TRIzol® reagent by passing the cell up and down through a pipette, transferred and incubated the lysate to a microcentrifuge tube for 5 min at room temperature.

3. Added 0.2 ml of chloroform, vigorously shake the tube by hand for 15 sec., and incubate at room temperature for 2-3 minutes.

4. Centrifuged at 12,000 rpm 4°C for 15 minutes and transfer the aqueous phase to a fresh tube.

5. Added 0.5 ml of isopropyl alcohol, incubated at room temperature for 10 min, and centrifuged 12,000 rpm at 4°C for 10 minutes.

6. Removed the supernatant, washed the RNA pellet with 1 ml of 75% ethanol, mixed by vortexing, separated the pellet by centrifugation 7,500 rpm at 4°C for 5 minutes.

7. Removed the supernatant and air-dry the RNA pellet (for 5-10 min).

8. Dissolved the pellet in DEPC-treated water, incubated at 55-60°C for 10 min, and stored at -70°C until use.

The amount of RNA in the sample was determined by spectrophotometer at 260 nm. The protein contamination in it was checked by the ratio of optical densities of the RNA sample at 260 nm and 280 nm which must be > 1.8.

2.4.2 Preparation of complementary DNA (cDNA) by reverse transcription-polymerase chain reaction

One µg of total RNA was reverse transcribed to cDNA synthesis using Improme II™ reverse transcription system reagent with oligo$(dT)_{15}$ primer following the manufacturer protocol.

2.4.3 Determine mRNA expression of melanogenesis by PCR

PCR was performed using gene-specific primers (table 1) for tyrosinase, TRP-1, TRP-2 and MITF. The beta-actin gene was used as the internal control. PCR was carried out in a 50 µl reaction mixture containing PCR buffer, 1 µl cDNA of tyrosinase, TRP-1,

TRP-2 and MITF, 2 μM $MgCl_2$, 0.1 mM mixed dNTP, 0.2 μM of each primers and 1 unit of Taq® DNA polymerase. The PCR conditions were: 94°C for 4 minutes., followed by 20 cycles for TRP-1, TRP-2 and beta-actin, 25 cycles for tyrosinase and MITF of 1 minute. denaturation at 94°C, 1 minute. annealing at appropriate Tm, 1 min extension at 72°C, and finally 4 min extension at 72°C. PCR products were analyzed by electrophoresis in 1.5% agarose gel at 100 V. The gel was stained with ethidium bromide in 1xTBE buffer. PCR products were imaged and determined its density by gel documentation. The density of the PCR products were expressed as % of internal control (ratio of the band density divided by that of the housekeeping gene (beta-actin) x 100)

Table 1: The gene-specific primers used for RT-PCR

Gene	Primer sequences
Beta actin	Forward: 5'-GTG GGC CGC CCT AGG CAC CAG-3' Reverse: 5'-GGA GGA AGA GGA TGC GGC AGT-3'
Tyrosinase	Forward: 5'-GGC CAG CTT TCA GGC AGA GGT-3' Reverse: 5'-TGG TGC TTC ATG GGC AAA ATC-3'
TRP-1	Forward: 5'-GCT GCA GGA GCC TTC TTT CTC-3' Reverse: 5'-AAG ACG CTG CAC TGC TGG TCT-3'
TRP-2	Forward: 5'-GGA TGA CCG TGA GCA ATG GCC-3' Reverse: 5'-CGG TTG TGA CCA ATG GGT GCC-3'
MITF	Forward: 5'-GTA TGA ACA CGC ACT CTC TCG A-3' Reverse: 5'-CTT CTG CGC TCA TAC TGC TC-3'

2.5 Determination of Melanocyte dendricity assay

1. Mouse B16F10 melanoma cells were plated at density 1×10^3 cells per dish and cultured for 24 hours in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum.

2. Cells were treated with media (untreated control), various concentrations of α-MSH (positive control) and various concentrations of xanthoxylin for 72 hours.

3. After this incubation for 72 hours the dendrites of cells were seen under microscope and photograph the pictures.

3. Statistical analysis

Mean plus or minus standard error of mean (mean ± S.E.M) were calculated; statistical analysis for comparisons of results was performed by one-way ANOVA followed by Tukey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 16. Value of p-value < 0.05 and p-value < 0.01 were considered significant.

CHAPTER IV

RESULTS

1. The effect of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells

To determine the effect of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells, 0.2% DMSO and α-MSH were used as the negative and the positive control, respectively. The concentration of α-MSH used in this study was first identified by varying its concentrations for determining its effect on melanogenesis. Alpha-MSH at the concentrations 0.001, 0.01, 0.1, 1 and 10 μM increased melanin content in B16F10 cells to 2.46, 3.08, 3.14, 3.56, and 3.59 folds when compared to the untreated cells (Fig. 17 and Fig. 18). Ten nM of α-MSH was chosen to use as the positive control.

Xanthoxylin at the concentrations 3.125, 6.25, 12.5, 25 and 50 μg/ml increased melanin content to 1.27, 1.53, 2.69, 6.51 and 8.18 folds when compared to the untreated or negative control (Fig. 19 and Fig. 20). It significantly stimulated melanogenesis in a concentration dependent manner.

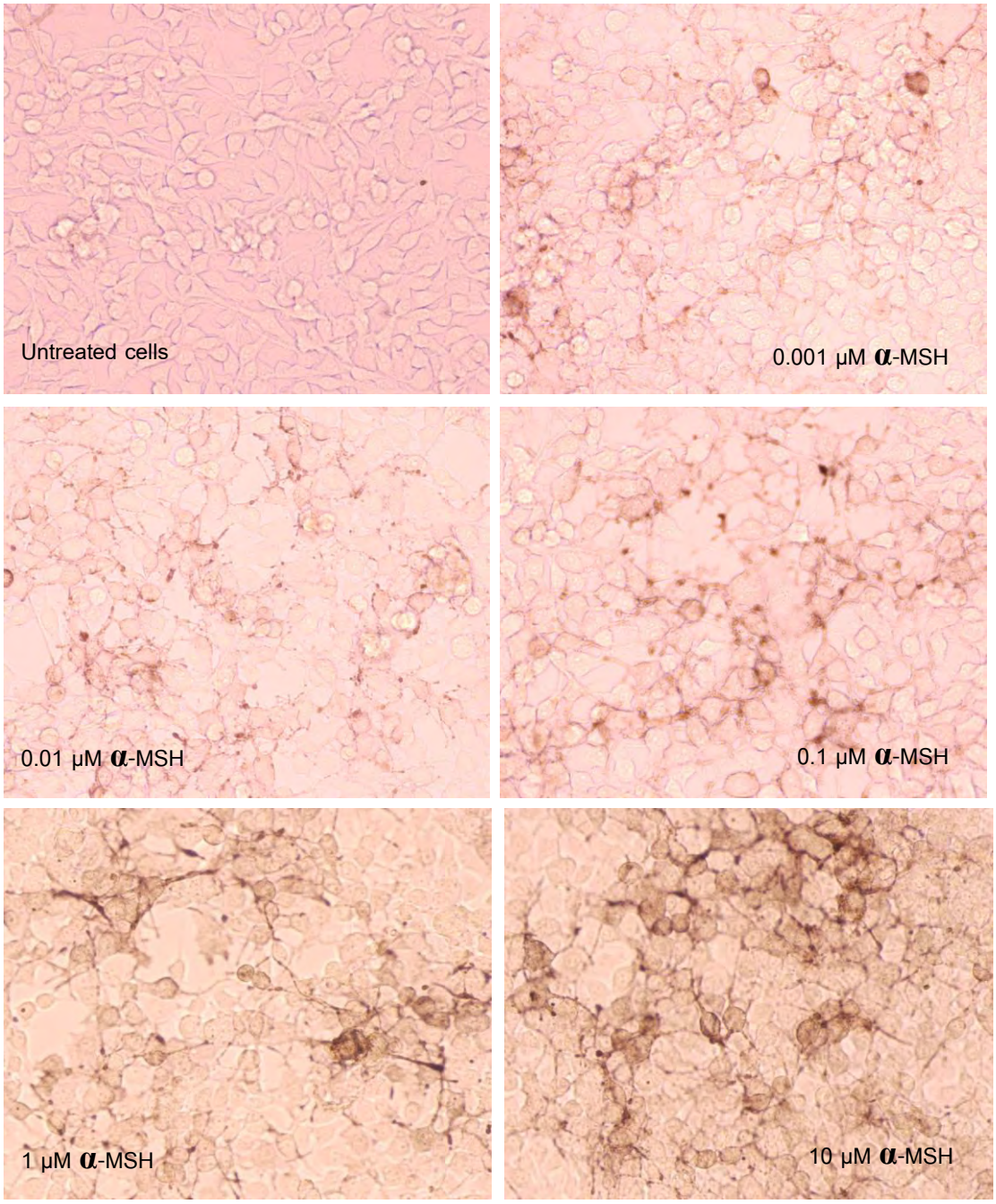


Figure 17: A representative results of the effect of α-MSH on melanin synthesis in mouse B16F10 melanoma cells under light microscope (10X); (from 3 independent experiments; n=3).

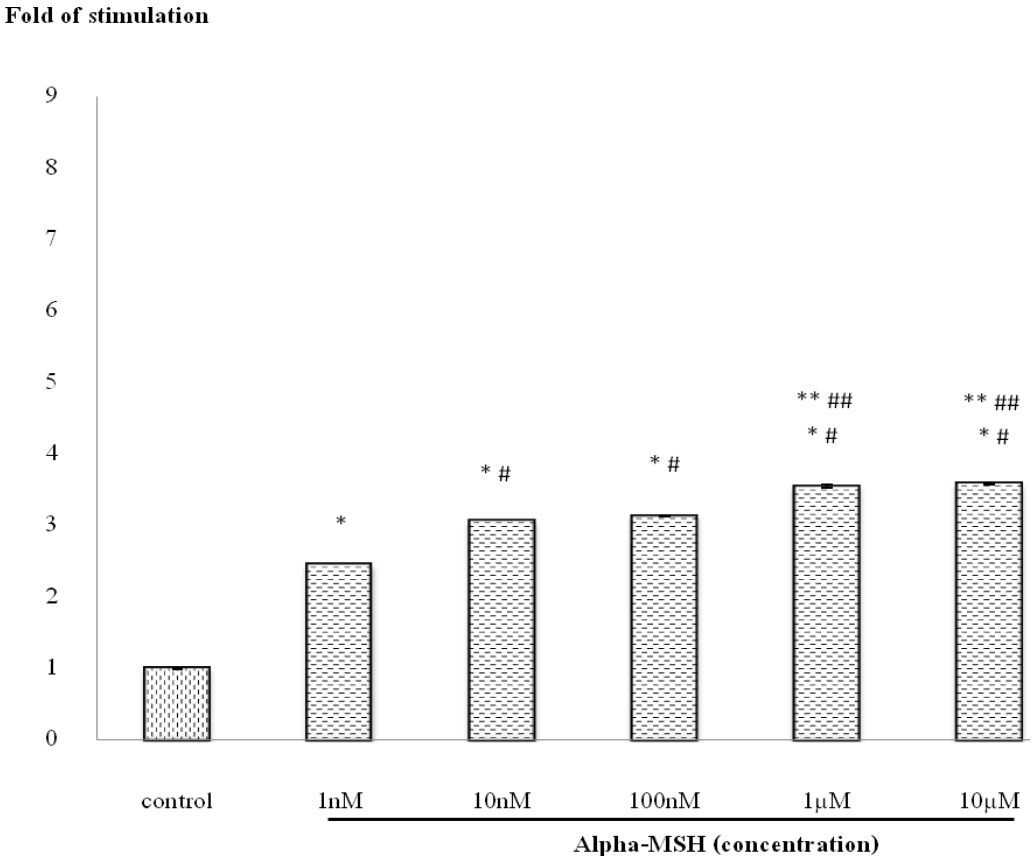


Figure 18: Effect of α-MSH on melanin synthesis. Mouse B16F10 melanoma cells were treated with α-MSH at the concentrations of 0.001, 0.01, 0.1, 1 and 10 μM for 72 hours. Melanin content in the treated cells were dissolved in 2 M sodium hydroxide. The folds of stimulation were determined from the absorbance at 405 nm. The data are presented as the mean ± S.E.M from 2 independent experiments.

* $p < 0.01$, statistically significant difference from control.

# $p < 0.01$, statistically significant difference from α-MSH 1 nM.

** $p < 0.01$, statistically significant difference from α-MSH 10 nM.

$p < 0.01$, statistically significant difference from α-MSH 100 nM.

° $p < 0.01$, statistically significant difference from α-MSH 1μM.

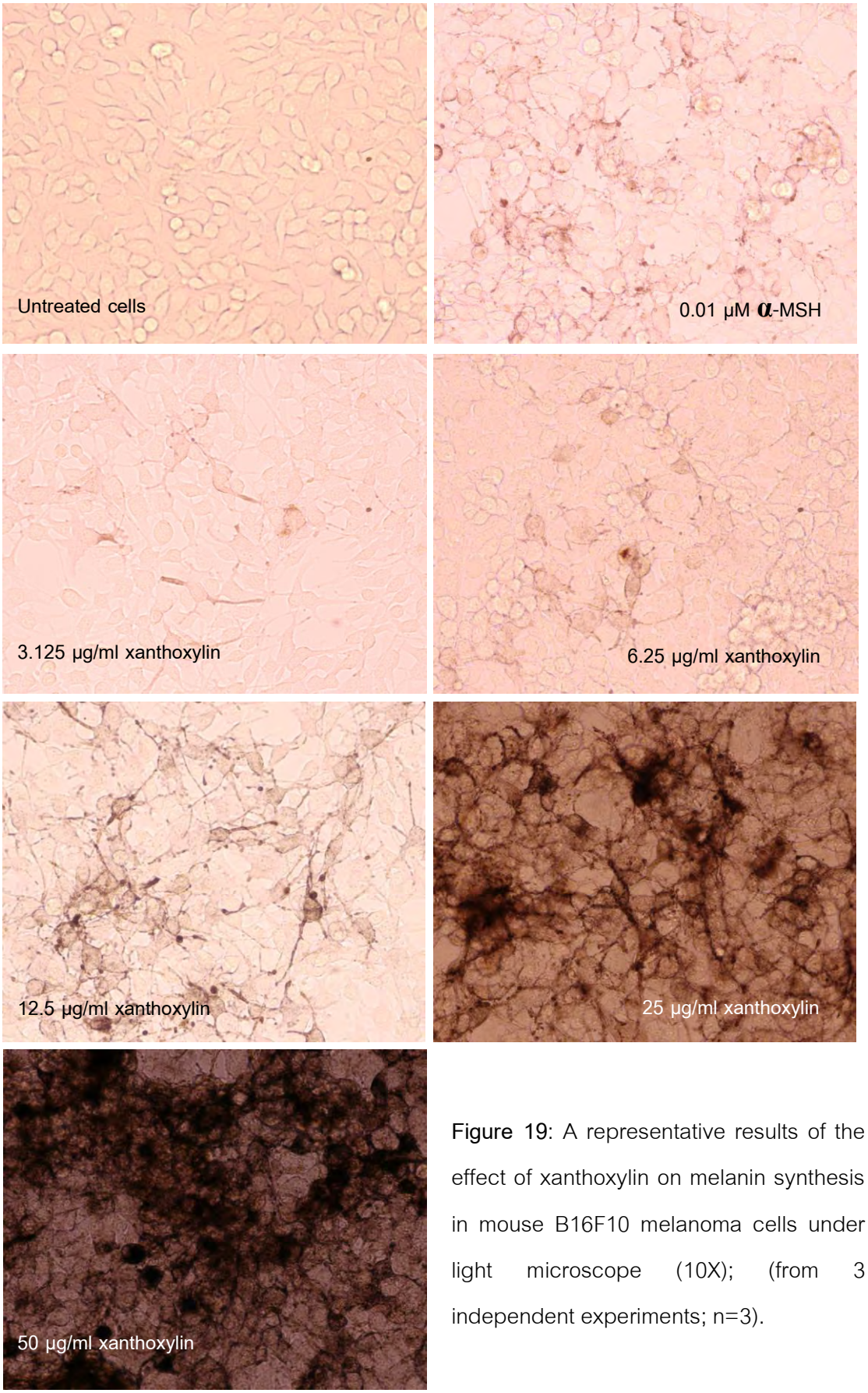


Figure 19: A representative results of the effect of xanthoxylin on melanin synthesis in mouse B16F10 melanoma cells under light microscope (10X); (from 3 independent experiments; n=3).

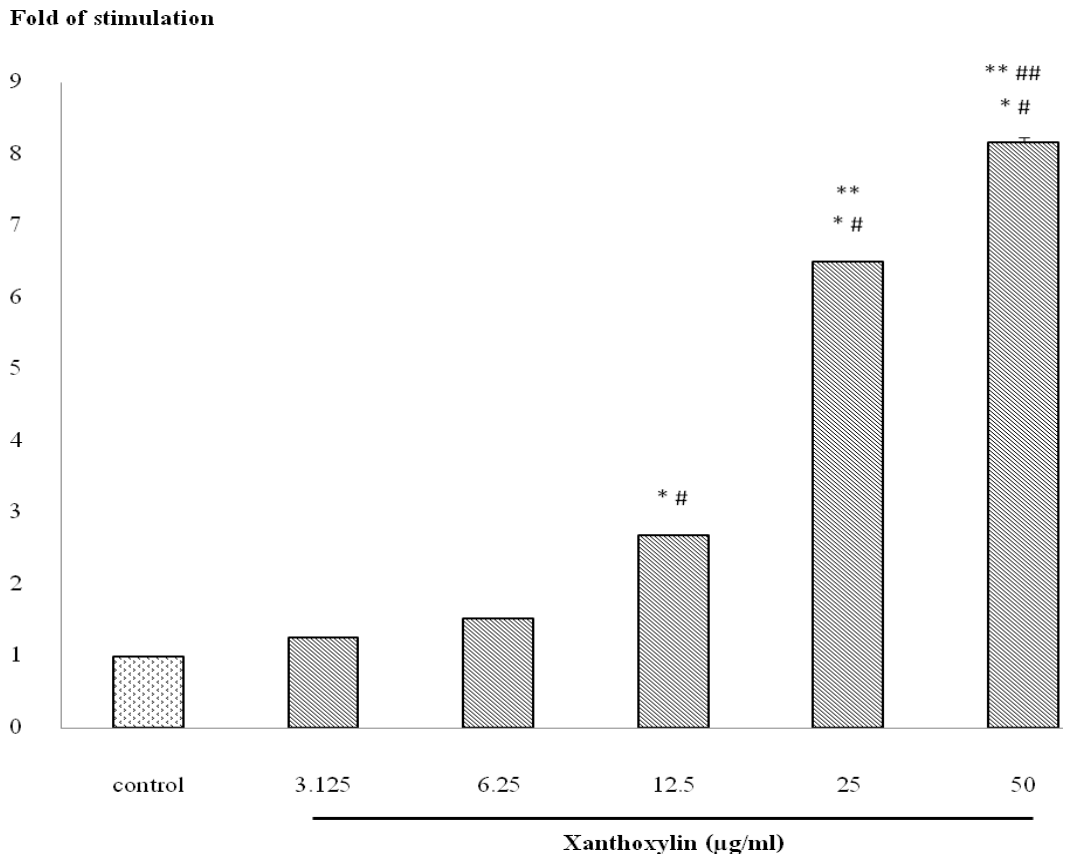


Figure 20: Effect of xanthoxylin on melanin synthesis. Mouse B16F10 melanoma cells were treated with xanthoxylin at the concentrations of 3.125, 6.25, 12.5, 25 and 50 µg/ml for 72 hours. Melanin content in the treated cells were dissolved in 2 M sodium hydroxide. The folds of stimulation were determined from the absorbance at 405 nm. The data are presented as the mean ± S.E.M from 4 independent experiments (n=4).

* $p < 0.01$, statistically significant difference from control.

# $p < 0.01$, statistically significant difference from xanthoxylin 6.25 µg/ml.

** $p < 0.01$, statistically significant difference from xanthoxylin 12.5 µg/ml.

$p < 0.01$, statistically significant difference from xanthoxylin 25 µg/ml.

2. Effect of xanthoxylin on cell viability

The cytotoxic or proliferative effect of xanthoxylin during assaying melanin content was also elucidated by resazurin assay. Mouse B16F10 cells were treated with xanthoxylin for 72 hours. The viability of the xanthoxylin treated cells, at the concentrations of 3.125, 6.25, 12.5 and 50 µg/ml, were 112.7%, 105.75%, 105.10%, and 87.30%, respectively when compared to the untreated control (Fig. 21). Xanthoxylin at the concentration 3.125 µg/ml significantly increased cell viability, but it significantly decreased cell viability at 50 µg/ml.

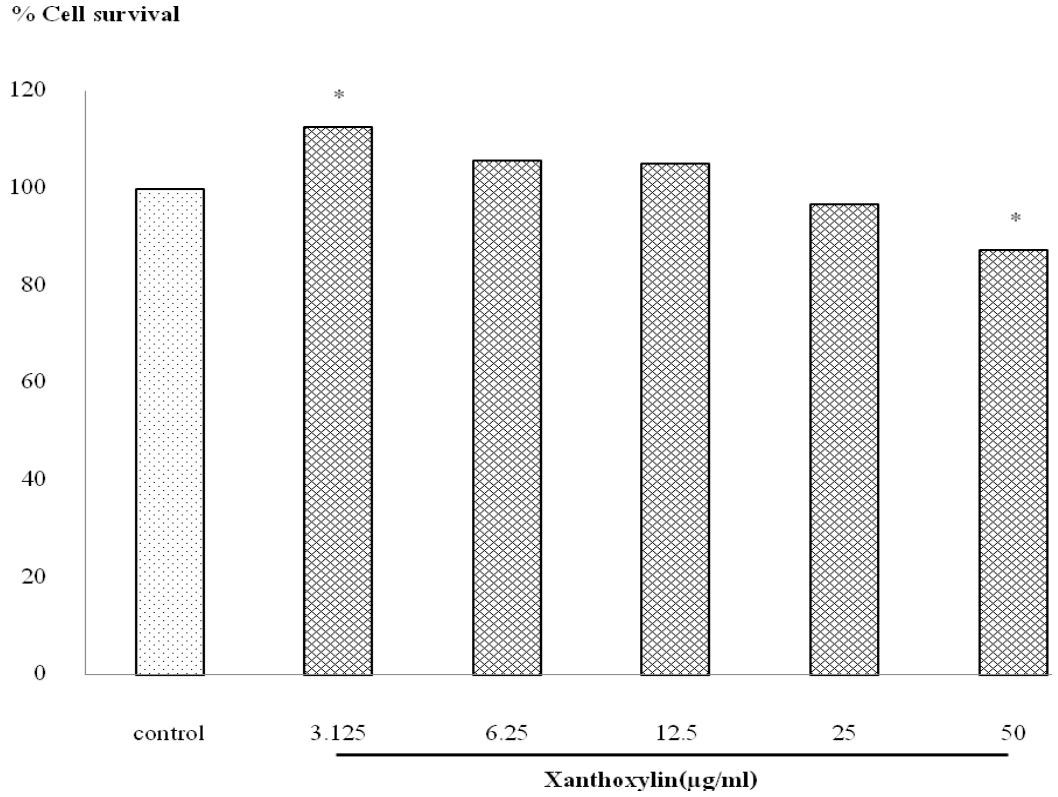


Figure 21: Effect of xanthoxylin on cell viability. Mouse B16F10 melanoma cells were treated with xanthoxylin at the concentrations of 3.125, 6.25, 12.5, 25 and 50 µg/ml for 72 hours. Viability of the treated cells was determined by staining with 1 mg/ml resazurin solution at 37°C for 4 hours. The percentage of cell survival was calculated from the absorbance at 570 and 600 nm; (n=4). The results are presented as the mean ± S.E.M from 4 independent experiments (n=4). * $p < 0.01$, statistically significant difference from control.

3. Mechanisms of xanthoxylin on induction of melanogenesis

To investigate the effect of xanthoxylin on signaling pathways involved in melanogenesis, 5 μM the inhibitors of the following kinases; PKA (H-89), PKB (LY294002), PKC (Ro-32-0432) and MEK1(PD98059), were used to inhibit signaling pathways involved in melanogenesis in mouse B16F10 melanoma cells. These inhibitors at the concentrations of 1, 5 and 10 μM were screened for their optimal concentrations to use in this study. It was found that at 5 μM of these inhibitors was the suitable concentration because it inhibited xanthoxylin effect higher than at 1 μM with no effect on cell viability, while these inhibitors at 10 μM reduced cell viability (data not shown). The cells were pretreated cells with each inhibitor at 37 ^{o}C for 1 hour and then treated with 25 μg/ml xanthoxylin at 37^{o}C for 72 hours. Ten nM α-MSH was used as the positive control. Effect of the protein kinase inhibitors on xanthoxylin-induced melanogenesis was determined by measuring melanin content in the treated cells. These inhibitors decreased the stimulatory effect of xanthoxylin from 8.43 folds to 3.77, 5.67, 7.12 and 7.28 folds for PKA, PKC, PKB and MEK1 inhibitors, respectively. They reduced the percentage of xanthoxylin-stimulated melanin content from 100% in non-pretreated cells to 44.66%, 67.25%, 84.39% and 86.28% in PKA, PKC, PKB and MEK1 inhibitor-pretreated cells respectively. These finding suggested that the mechanisms of xanthoxylin-induced melanogenesis in mouse B16F10 melanoma cells mainly involved protein kinase A signaling. However, the results also shown that xanthoxylin may partially induced melanin synthesis via PKC, PKB and MAPK signaling pathways.

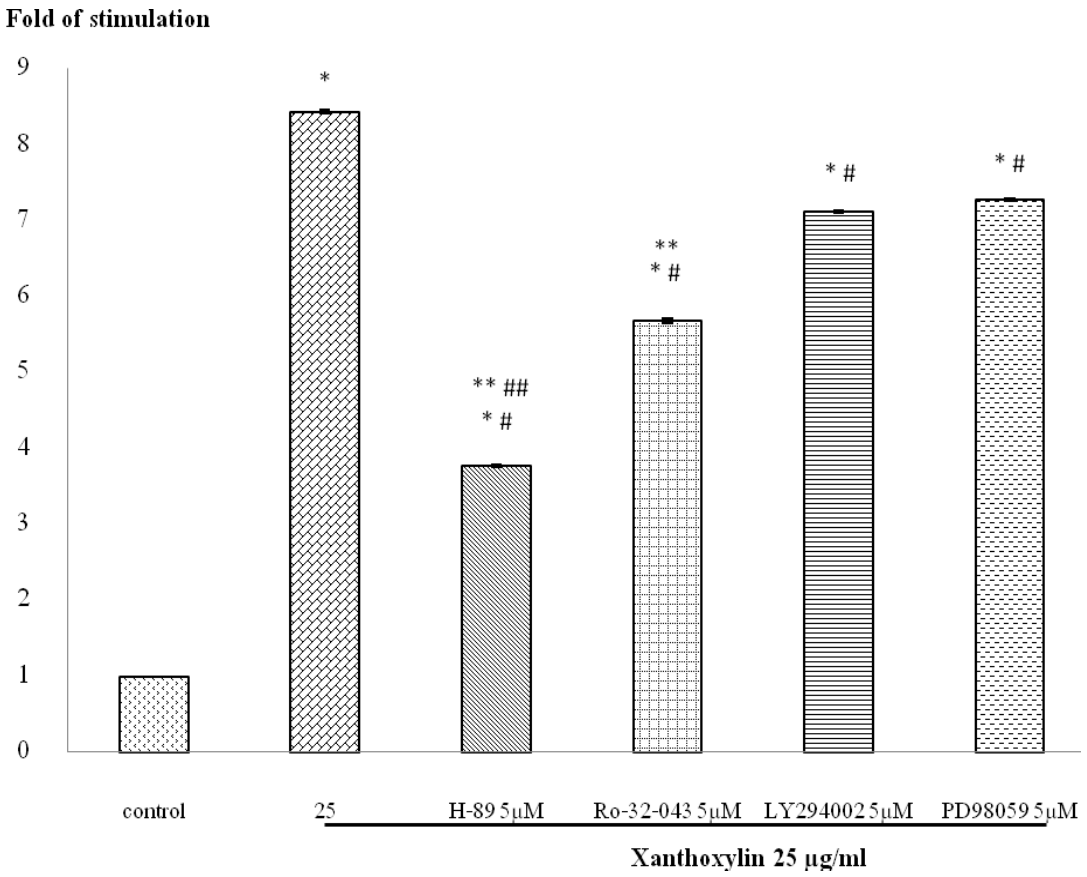


Figure 22: Effect of protein kinase inhibitors on xanthoxylin-induced melanogenesis. Mouse B16F10 melanoma cells were pretreated with 5 µM PKA, PKB, PKC and MEK1 inhibitors at 37°C for 1 hour and then treated with 25 µg/ml xanthoxylin at 37°C for 72 hours. Melanin content in the treated cells were dissolved in 2 M sodium hydroxide. The folds of stimulation were determined from the absorbance at 405 nm. The data are presented as the mean ± S.E.M from 4 independent experiments (n=4).

* $p < 0.01$, statistically significant difference from control.

# $p < 0.01$, statistically significant difference from xanthoxylin 25 µg/ml alone.

** $p < 0.01$, statistically significant difference from cells were pretreated with PD98059.

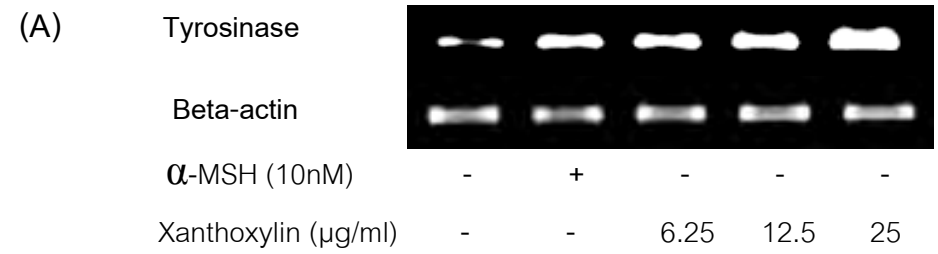
$p < 0.01$, statistically significant difference from cells were pretreated with Ro-32-043.

4. Effect of xanthoxylin on the mRNA expression of proteins involve in melanogenesis

MITF is the transcription factor that plays role in melanogenesis by regulating the mRNA expression of tyrosinase, TRP-1 and TRP-2 proteins. Tyrosinase is the essential enzyme that converts tyrosine to melanin, while TRP-1 and TRP-2 proteins function in maintaining and stabilizing tyrosinase at the melanosomal membrane. The effect of xanthoxylin on the mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 in was investigated in this study. Mouse B16F10 melanoma cells were treated with 6.25, 12.5 and 25 µg/ml xanthoxylin at 37 ^{o}C for 72 hours. The mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 was determined by RT-PCR with specific primers to these genes. Ten nM α-MSH was used as the positive control.

Xanthoxylin significantly increased tyrosinase expression in a dose dependent manner (Fig. 23). It increased the ratio of tyrosinase to beta-actin expression from 1.06 in the untreated control to 1.47, 1.78 and 2.01 in 6.25, 12.5 and 25 µg/ml xanthoxylin-treated cells, respectively. It also significantly induced MITF expression at all concentrations used in the study (Fig.24). It increased the ratio of MITF to beta-actin expression from 0.26 in the untreated control to 0.93, 1.11 and 1.13 in 6.25, 12.5 and 25 µg/ml xanthoxylin-treated cells, respectively. However, it didn't have any effect on TRP-1 and TRP-2 expression, as shown in Fig. 25 and Fig. 26.

(A)



(B)

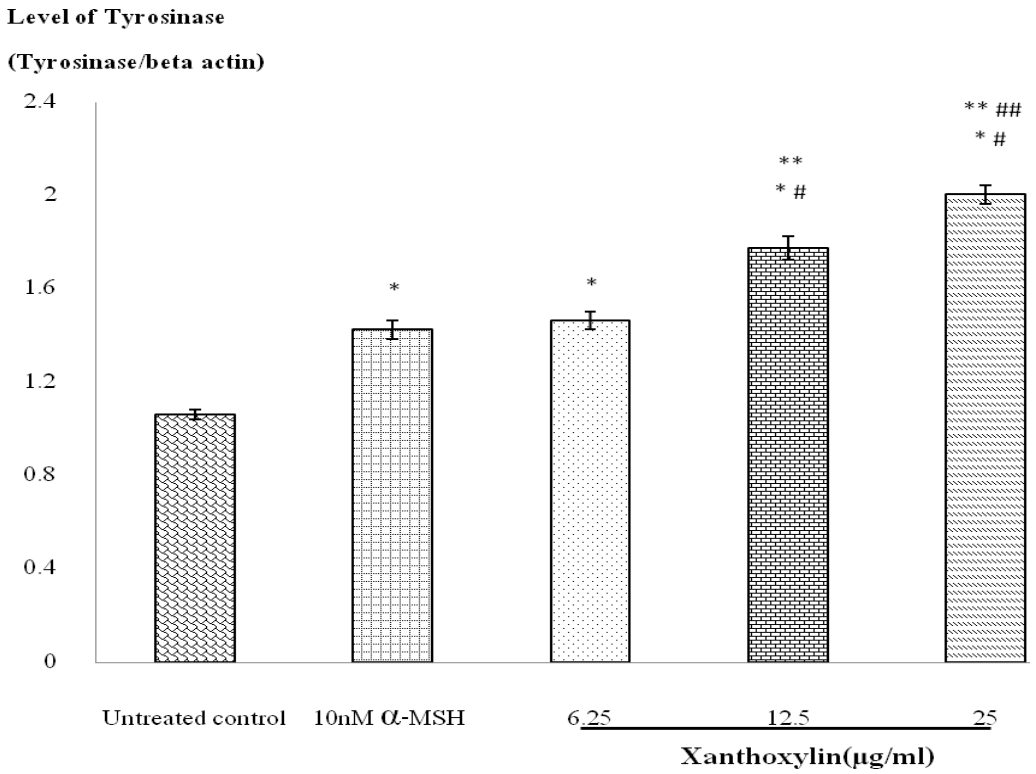


Figure 23: Effect of xanthoxylin on tyrosinase expression. B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of tyrosinase in these cells was identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of tyrosinase to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from α-MSH 10 nM. ** $p < 0.05$, statistically significant difference from xanthoxylin 6.25 μg/ml. ## $p < 0.05$, statistically significant difference from xanthoxylin 12.5 μg/ml.

(A)

α-MSH(10nM)	-	+	-	-	-
Xanthoxylin(µg/ml)	-	-	6.25	12.5	25

(B)

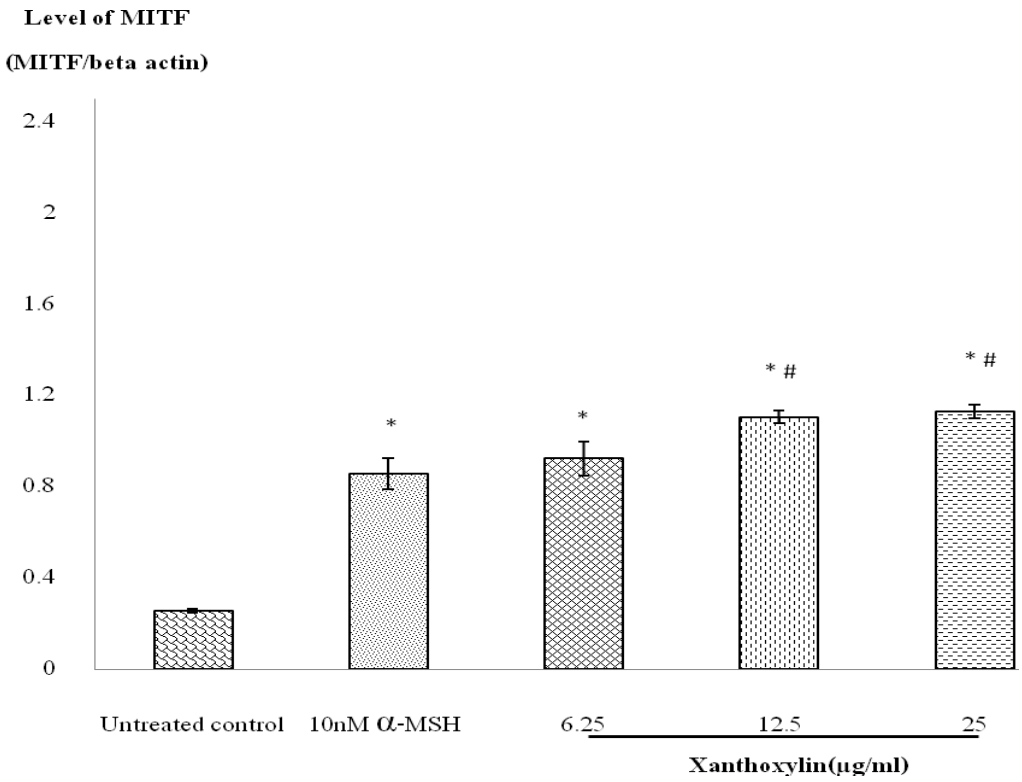


Figure 24: Effect of xanthoxylin on MITF expression. B16F10 cells were treated with 6.25, 12.5 and 25 µg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of MITF in these cells were identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of MITF to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from α-MSH 10 nM. ** $p < 0.05$, statistically significant difference from xanthoxylin 6.25 µg/ml. ## $p < 0.05$, statistically significant difference from xanthoxylin 12.5µg/ml.

(A)

α-MSH (10nM)	-	+	-	-	-
Xanthoxylin (μg/ml)	-	-	6.25	12.5	25

(B)

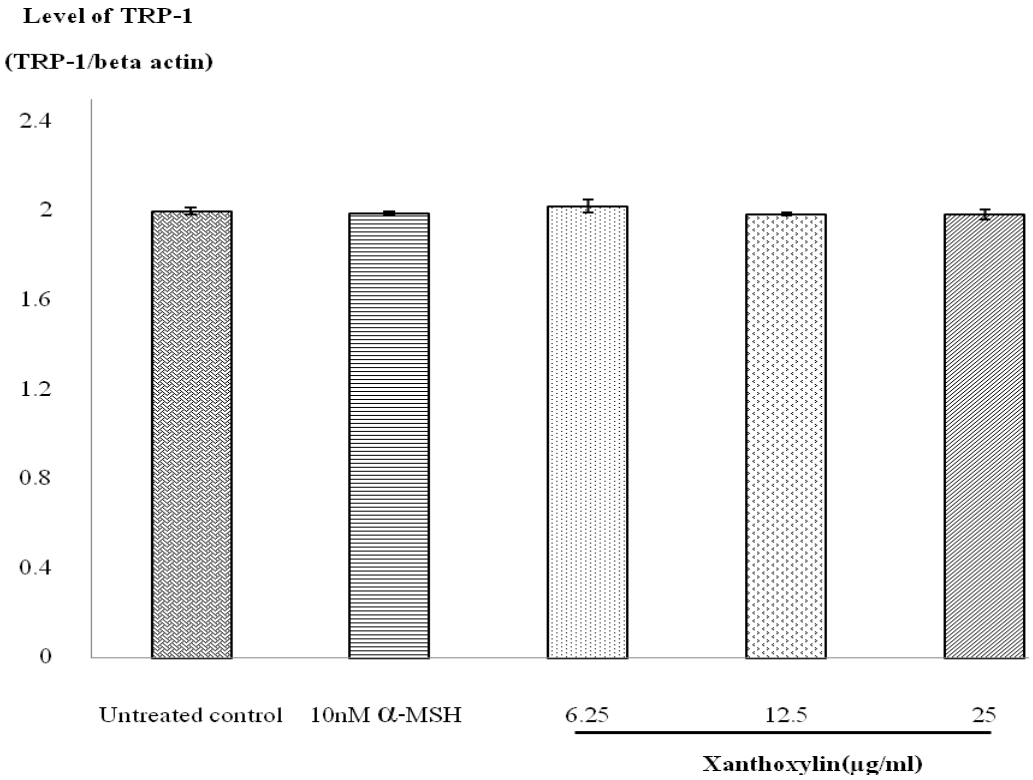


Figure 25: Effect of xanthoxylin on TRP-1 expression. B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of TRP-1 in these cells were identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of TRP-1 to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from α-MSH 10 nM. ** $p < 0.05$, statistically significant difference from xanthoxylin 6.25 μg/ml. ## $p < 0.05$, statistically significant difference from xanthoxylin 12.5 μg/ml.

(A)

(B)

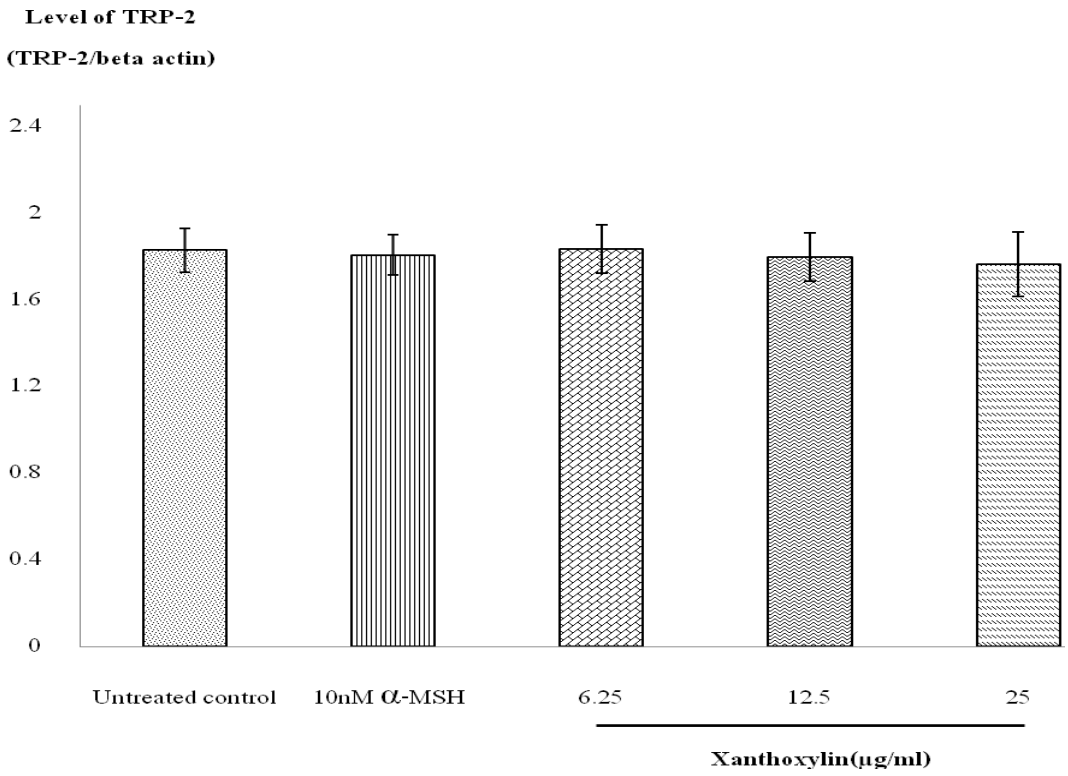


Figure 26: Effect of xanthoxylin on TRP-2 expression. B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of TRP-2 in these cells were identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of TRP-2 to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from α-MSH 10 nM. ** $p < 0.05$, statistically significant difference from xanthoxylin 6.25 μg/ml. ## $p < 0.05$, statistically significant difference from xanthoxylin 12.5 μg/ml.

5. Effects of protein kinase inhibitors on xanthoxylin-induced tyrosinase and MITF expression

Inhibitors of PKA, PKB, PKC and MEK1 were used to investigate the signaling pathways involved in xanthoxylin-induced tyrosinase and MITF expression. Mouse B16F10 melanoma cells were pretreated with 5 μM of inhibitors of the following protein kinases; PKA (H-89), PKB (LY294002), PKC (Ro-32-0432) and MEK1 (PD98059), at 37°C for 1 hour, and then treated with xanthoxylin at 37°C for 72 hours. The mRNA expression of tyrosinase and MITF was determined by RT-PCR with specific primers to these genes.

The PKA inhibitor, H-89, decreased xanthoxylin-induced tyrosinase and MITF expression, as demonstrated in Fig. 27 and Fig. 28. It significantly reduced xanthoxylin-induced mRNA expression of tyrosinase at all concentrations of xanthoxylin (6.25, 12.5 and 25 μg/ml) used in the study (Fig. 27). It also decreased the effect of xanthoxylin on MITF expression (Fig. 28). It significantly reduced MITF expression in the 25 μg/ml xanthoxylin-treated cells.

Effects of the other protein kinase inhibitors were studied on 25 μg/ml xanthoxylin-treated cells. The PKC inhibitor, Ro-32-0432, significantly reduced the xanthoxylin-induced tyrosinase expression, but it didn't significantly decreased the effect of xanthoxylin on MITF expression. The PKB and MEK1 inhibitors, LY294002 and PD98059, didn't reduced neither tyrosinase nor MITF expression in xanthoxylin-treated cells (Fig. 27 and Fig.28)

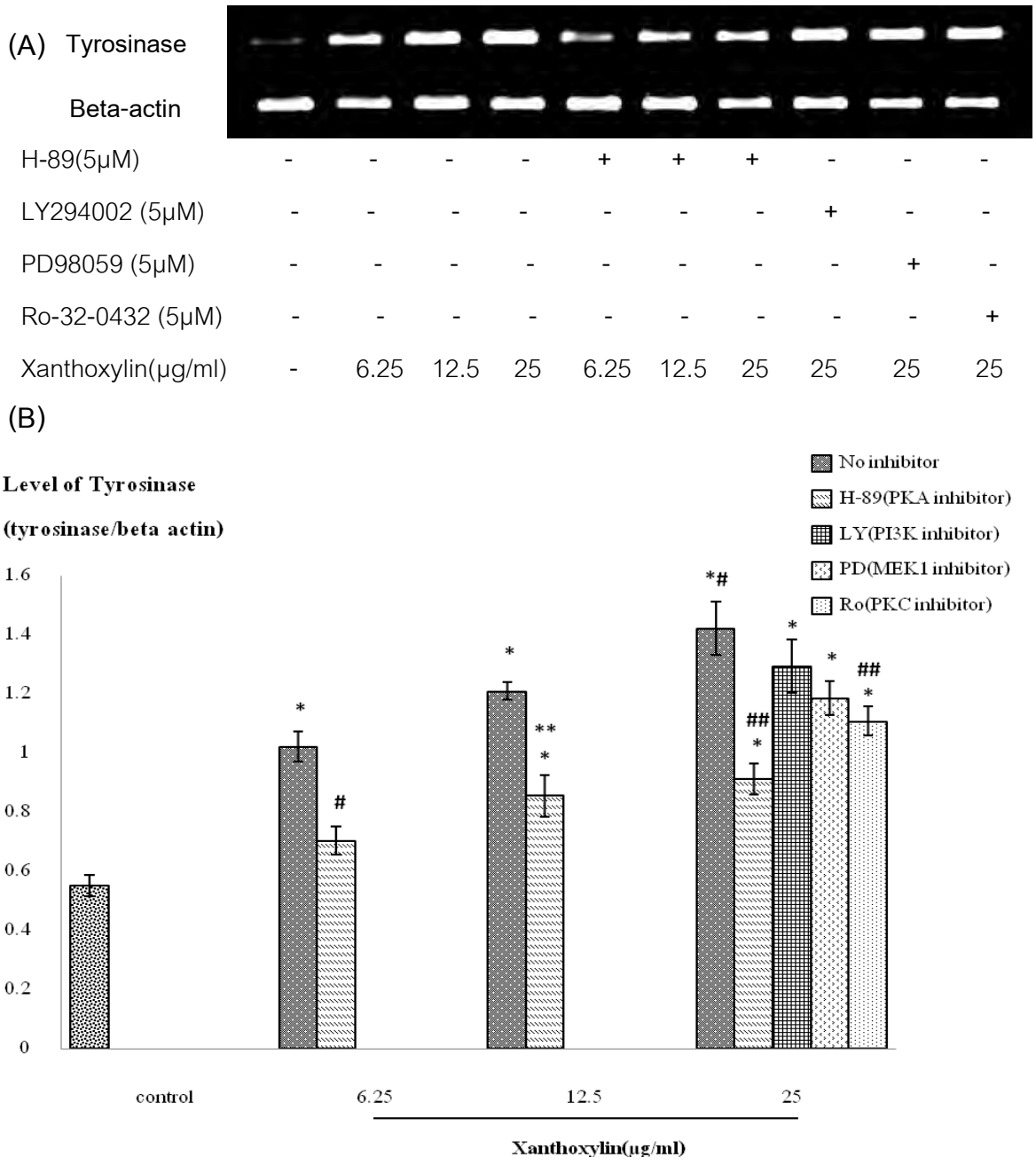


Figure 27: Effect of protein kinase inhibitors on xanthoxylin-induced tyrosinase expression. B16F10 cells were pretreated with 5 μM protein kinase inhibitor for 1 hour and then treated with xanthoxylin for 72 hours. The mRNA expression of tyrosinase in the treated cells was identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of tyrosinase to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from xanthoxylin 6.25 μg/ml alone. ** $p < 0.05$, statistically significant difference from xanthoxylin 12.5 μg/ml alone. ## $p < 0.05$, statistically significant difference from xanthoxylin 25 μg/ml alone.

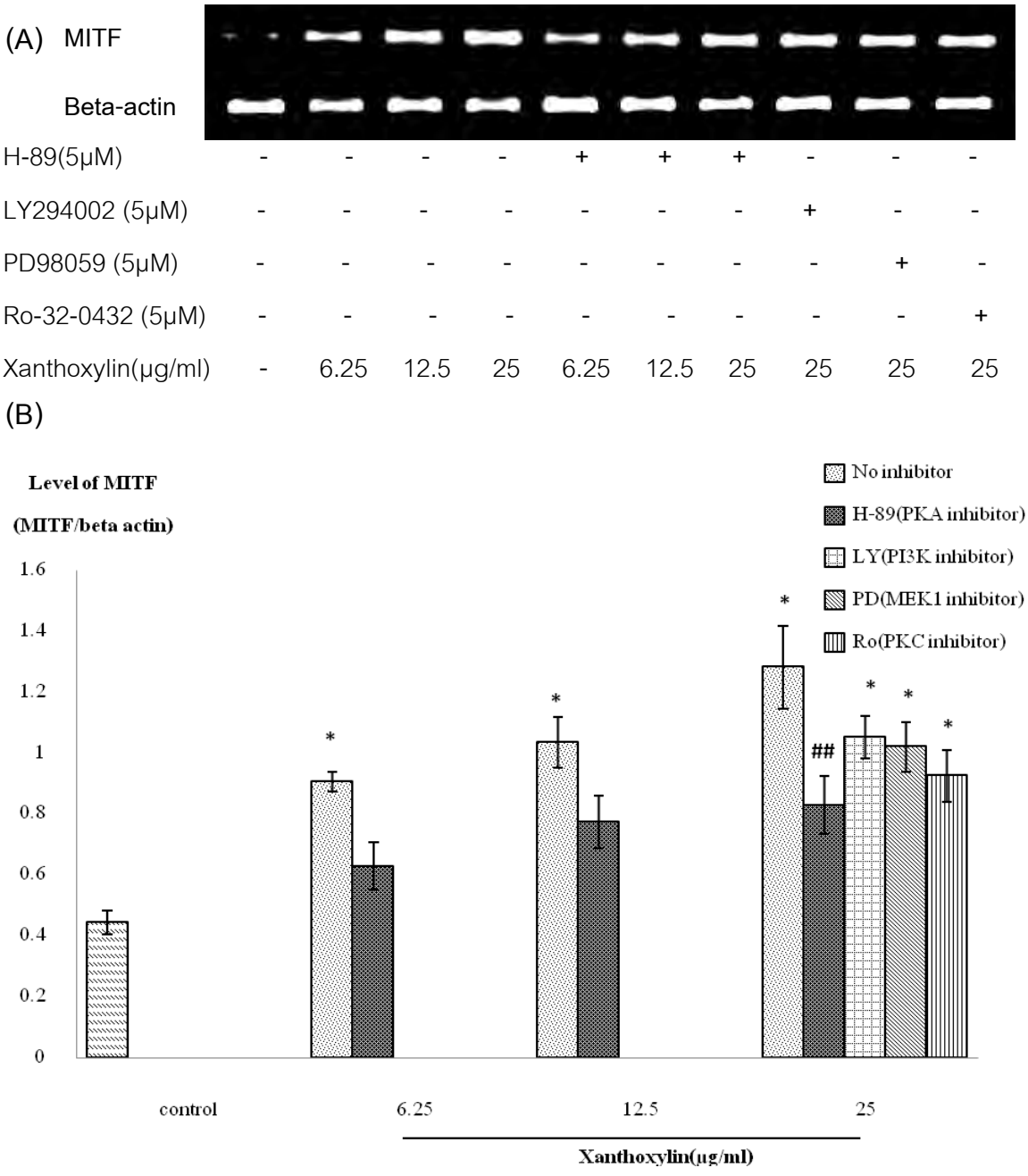


Figure 28: Effect of protein kinase inhibitors on xanthoxylin-induced MITF expression. B16F10 cells were pretreated with 5 μg/ml protein kinase inhibitor for 1 hour and then treated with xanthoxylin for 72 hours. The mRNA expression of MITF in the treated cells were identified by RT-PCR with specific primers. (A) Representative PCR products from one of the three independent experiments. (B) Densitometric analysis the PCR products. The data were represented as the density ratio of MITF to β-actin. The values are the mean ± S.E.M of three independent experiments (n=3). * $p < 0.05$, statistically significant difference from control. # $p < 0.05$, statistically significant difference from xanthoxylin 6.25 μg/ml alone. ** $p < 0.05$, statistically significant difference from xanthoxylin 12.5 μg/ml alone. ## $p < 0.05$, statistically significant difference from xanthoxylin 25 μg/ml alone.

6. Effect of xanthoxylin on dendricity

Melanocyte dendricity is important in melanosome transfer to keratinocytes. The effect of xanthoxylin on melanocyte was also investigated. Mouse B16F10 melanoma cells were treated with xanthoxylin at the concentrations of 3.125, 6.25, 12.5, 25 and 50 µg/ml at 37°C for 72 hours. The untreated and 10 nM α-MSH-treated cells were used as the negative and positive control, respectively. The dendricity of the treated cells were observed under a light microscope. Xanthoxylin, at all concentrations, induced an increase in the number of B16F10 cells with more than two dendrites (Fig. 29: C-G), while most of the untreated cells had only two dendrites (Fig. 29: A). Ten nM α-MSH also increased melanocyte dendricity (Fig. 29: B).

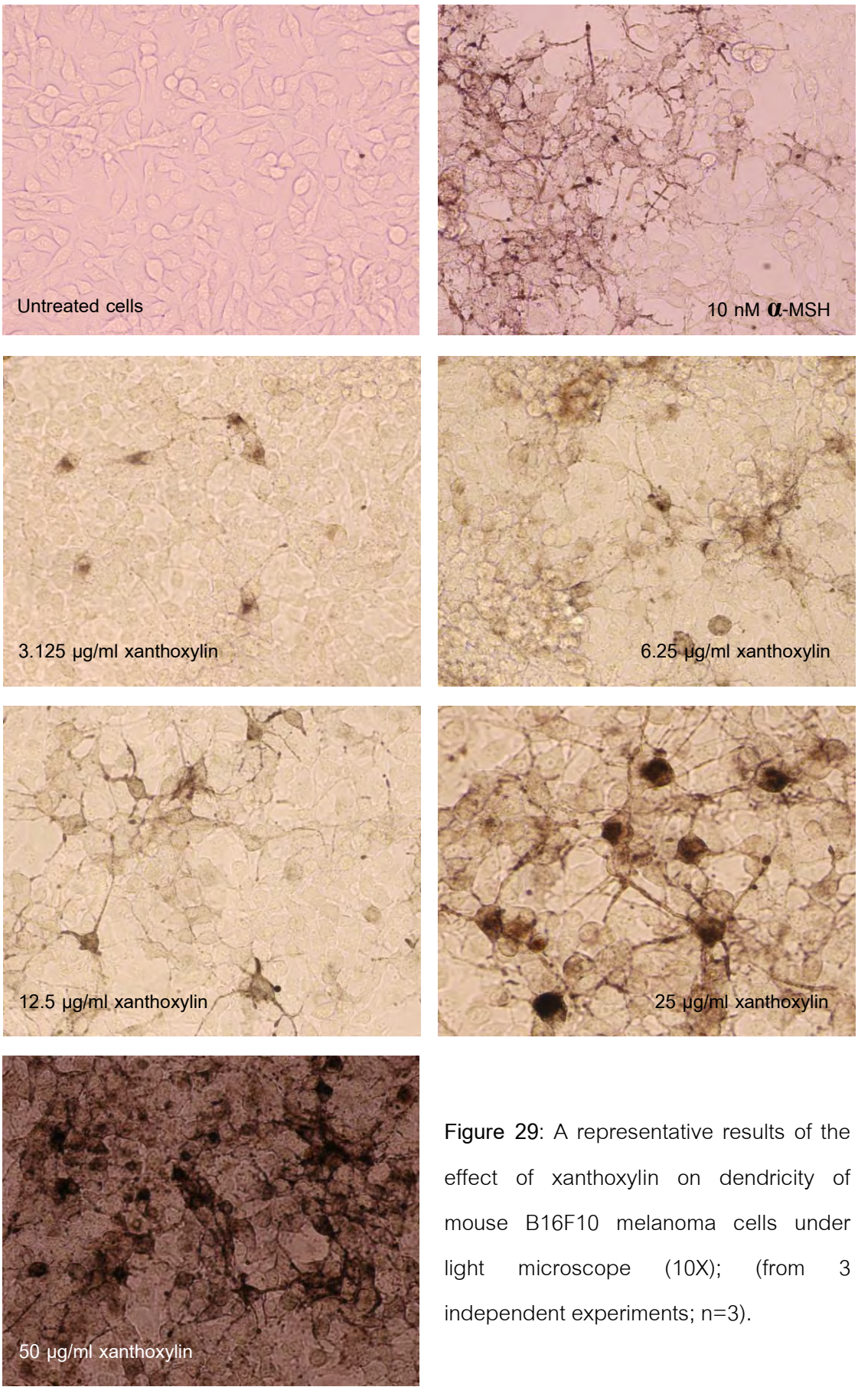


Figure 29: A representative results of the effect of xanthoxylin on dendricity of mouse B16F10 melanoma cells under light microscope (10X); (from 3 independent experiments; n=3).

CHAPTER V

DISCUSSION AND CONCLUSION

Melanogenesis is a complex process that includes melanin synthesis in melanosomes of melanocyte, the transport and the transfer of melanosomes through melanocyte dendrites to keratinocytes. Melanin plays an important role in protection skin against UV radiation. Stimulation of melanogenesis is counted as a protective mechanism to prevent DNA damage and skin cancer from UV radiation. Mealnin synthesis is enhanced by numerous agents such as UV radiation, α-MSH, forskolin, and IBMX but very few are used as skin enhancers for human skin due to weak effectiveness or undesirable side effects. Facultative pigmentation, more commonly skin tanning is occurred by exposure to UV radiation [115]. This radiation increases number of melanocytes, number and size of melanosomes, melanin content in melanosomes, and number of melanosomal dendrites. However UV-induced hyperpigmentation can lead to DNA damage and skin cancer. A highly effective and UV-less skin enhancer without skin damage may be beneficial. It has been demonstrated in this study that xanthoxylin purified fruits from *Zanthoxylum piperitum* or Japanese pepper may be one of the agents of purpose. Mouse B16F19 melanoma cells were used in this study because they are widely used as a cell model system to study the effects of various compounds on melanogenesis [116, 117, 118, 119]. These cells have a short population doubling time. They are easy to culture and have better survival rates than human melanocyte cells [120]. Xanthoxylin significantly increased melanin content in mouse B16F10 melanoma cells in a concentration dependent manner with little effect on cell viability. It increased not only melanin content in B16F10 cells but also dendricity of the cells which is important for transporting melanosomes to keratinocytes in melanogenesis process. These findings were similar to the previous study which demonstrated that quercetin (3,5,7,3',4'-pentahydroxyflavone) induced melanogenesis by increased melanin content and stimulated dendrites of melanocytes extended towards the adjacent keratinocytes in human epidermis culture model [121].

The numerous proteins are involved in melanogenesis. Among these, tyrosinase, TRP-1, and TRP-2 are essential for melanin synthesis. Expression of these three proteins is mainly regulated by a key transcription factor MITF and also by other several transcription factors and regulatory proteins or DNA elements [2]. MITF expression is also regulated by several transcription factors, regulatory proteins and DNA elements. Xanthoxylin increased melanin content was correlated to the increase of the mRNA expression of MITF and tyrosinase. It has also been reported that glycyrrhizin (GR) stimulates the melanogenesis in B16F10 cells by increased expression of MITF and tyrosinase [59, 122]. These results strongly suggest that xanthoxylin induces the increase in melanin synthesis by MITF-dependent activation of tyrosinase expression. However xanthoxylin didn't have any change in the mRNA expression of TRP-1 and TRP-2 which their genes are coordinately regulated with the tyrosinase gene. It has been known that tyrosinase, TRP-1 and TRP-2 genes are coordinately regulated by MITF transcription factor which binds to M-box sequences of these genes with different affinity [122, 123]. Moreover, there are several lines of evidence indicated that these genes are also regulated independently of each other as well as independently of MITF [124-126].

There are several signal pathways for melanogenesis regulation. Recent studies reveal that cAMP and PKC signaling pathways are critical for melanin synthesis [127]. PI3K-PKB pathway and p38-MAPK signaling cascade are also reported to be involved in the induction of melanogenesis [128]. The cAMP signaling pathway plays a key role in melanogenesis by increasing preexist tyrosinase enzyme activity as well as inducing the mRNA expression level of tyrosinase. The main mechanism of cAMP-induces melanogenesis involves the activation of PKA and CREB transcription factors by phosphorylation. Phosphorylated CREB then interacts with CBP to activate the expression of MITF which results in activation of tyrosinase gene transcription. Increased melanin content as well as MITF and tyrosinase mRNA expressions in B16F10 melanoma cells by xanthoxylin were inhibited by a PKA inhibitor H-89 in this study. These results suggest that cAMP signaling pathway through PKA activation is involved in xanthoxylin-induced stimulation of melanogenesis. Rosmarinic acid (a-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid) has also been reported to induce melanogenesis through

PKA activation. Its activity was inhibited by a PKA inhibitor H-89 [128]. However, H-89 couldn't completely block xanthoxylin effect. This raises the possibility that not only PKA but also other signaling mediators may be associated with xanthoxylin-induced the increase of melanin synthesis. PKA activation may be the main signaling pathway of xanthoxylin-increased melanin content as well as the expression of crucial proteins involve in melanin synthesis.

Some extracellular signals such as endothelin-1 (ET-1) induce increase in melanogenesis by activating PKC [82]. Active PKC activates tyrosinase by directly phosphorylating tyrosinase in melanosome. Similar to H-89 PKA inhibitor, an inhibitor of PKC Ro-32-0432, decreased the xanthoxylin-stimulated melanogenesis and the mRNA expressions of MITF and tyrosinase genes in the less extent than the effect of H-89 PKA inhibitor. These results suggest that xanthoxylin may also enhance melanin synthesis through PKC signaling. This needs further study to identify whether it is associated with cAMP/PKA pathway or not.

Inhibitors of PKB (LY294002) and MEK1 (PD98069) also slightly decreased xanthoxylin-increased melanin content and the mRNA expressions of MITF and tyrosinase genes. It has been reported that cAMP activates not only PKA activation but also other signaling pathways. These pathways include PI3K/AKT and PKB/GSK-3β pathways [2, 60]. It is possible that xanthoxylin may act through cAMP upstream of PKA, PKB, and MEK1 activation. It is considered that regulation of melanogenesis at the level of transcription is only the initial step; the other important step is posttranslational modification such as phosphorylation. Effect of xanthoxylin on proteins involved in melanogenesis and signaling molecules at the protein level should be investigated.

In conclusion, this is the first time to reveal the melanogenic effect of xanthoxylin as well as the molecular mechanisms of xanthoxylin-induced melanogenesis. The results in this study demonstrate that xanthoxylin increased melanin content and number of dendrites in B16F10 melanoma cells without effect on cell viability. It is possible that xanthoxylin induced melanin synthesis mainly via PKA activation to stimulate the expression of MITF transcription factor and tyrosinase (Fig. 30)

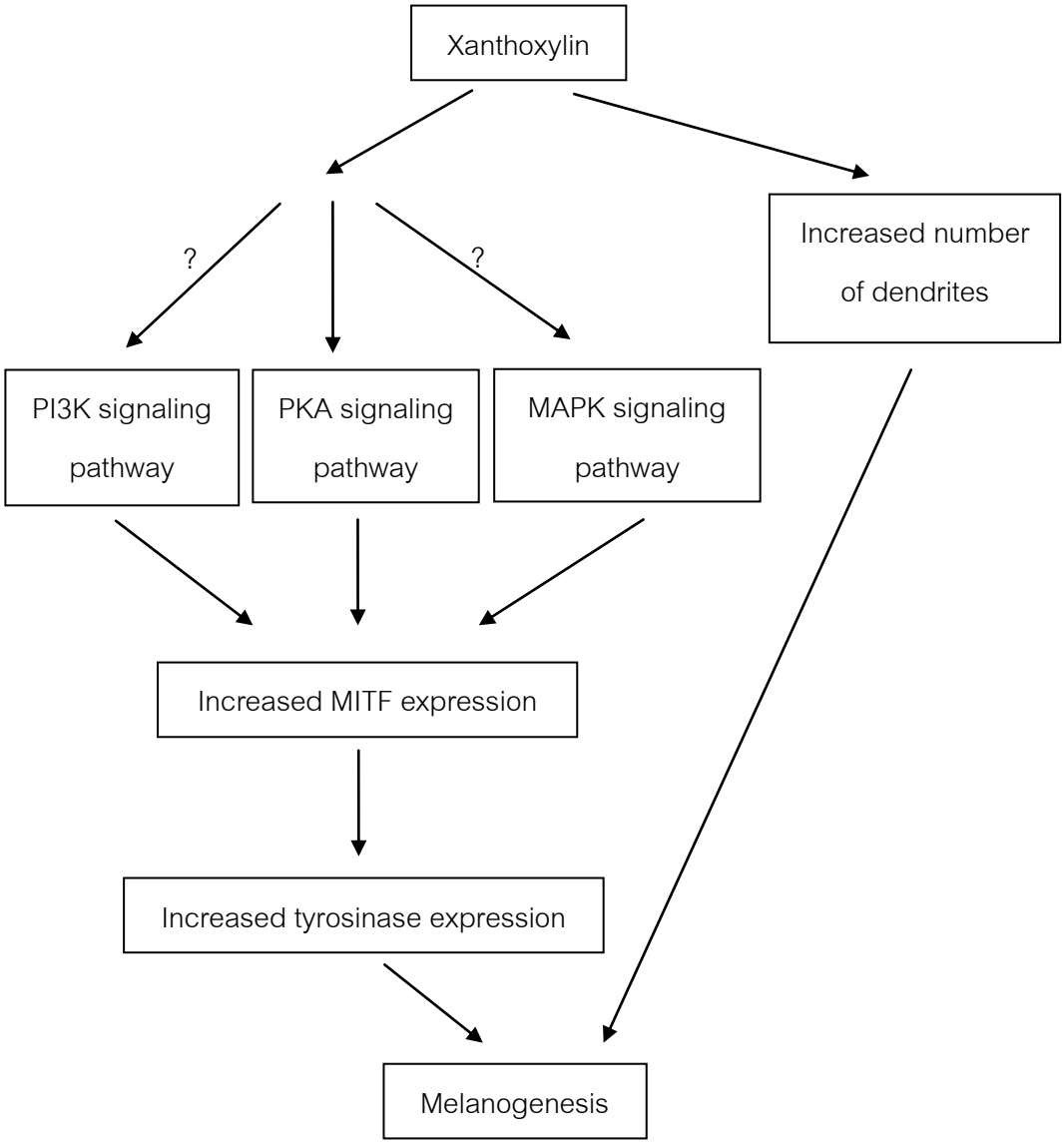


Figure 30: The effects of xanthoxylin on melanogenesis in mouse B16F10 melanoma cells.

Xanthoxylin may be a potential pharmacological agent useful for hypopigmentation-related diseases as well as for skin tanning.

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APPENDICES

APPENDIX A

Buffers and Reagents

1. DMEM stock solution 1 liter

DMEM	10.40	g
Sodium bicarbonate	3.70	g
Penicillin/Streptomycin	10.00	ml
ddH_2O	900.00	ml

Adjust pH to 7.2 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by filtering through a 0.45 membrane filter

2. Complete DMEM medium 200 ml

DMEM stock	180.00	ml
Fetal Bovine Serum	20.00	ml

3. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80.65	g
KCl	2.00	g
KH_2PO_4	2.00	g
Na_2HPO_4	11.50	g
ddH_2O	900.00	ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by autoclaving

4. 1.5% Agarose gel 100 ml

Agarose	1.50	g
1x TBE	80.00	ml

Add 1x TBE to 100 ml

Dissolve by heating and mixing until no granules of agarose are visible

5. NaOH 2M 100 ml

NaOH	11.688	g
H_2O	80.000	ml

Add H_2O to 100 ml

6. EDTA 0.5M pH 8.0 100 ml

EDTA	18.612	g
ddH_2O	80.000	ml

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 ml and Sterilized by autoclaving

7. 1x TE Buffered 100 ml

Tris-HCl 1M pH 8.0	1.0	ml
EDTA 0.5M pH 8.0	0.2	ml
ddH_2O	98.8	ml

Sterilized by autoclaving

8. 5x TBE Buffered 1 liter

Tris-base	54.0	g
Boric acid	27.5	g
EDTA 0.5M pH 8.0	20.0	ml

Sterilized by autoclaving

APPENDIX B

Results

Table 2: Effect of xanthoxylin on melanigenesis. B16F10 cells were treated with 3.125, 6.25, 12.5, 25 and 50 µg/ml xanthoxylin for 72 hours. Melanin content in the treated cells was determined and compared to the untreated control. The results are presented as the mean ± S.E.M from 4 independent experiments (n=4). * P<0.01, significantly when compared to untreated control. # P<0.01, significantly when compared to 12.5 µg/ml xanthoxylin.

Xanthoxylin (µg/ml)	N1		N2		N3		N4		Average folds of stimulation	% Stimulation of melanin content
	OD 405 nm	Folds of stimulation	OD 405 nm	Folds of stimulation	OD 405 nm	Folds of stimulation	OD 405 nm	Folds of stimulation		
Untreated control	0.164	1.00	0.165	1.00	0.162	1.00	0.165	1.00	1.0 ± 0.0008	100.00
3.125	0.213	1.30	0.205	1.25	0.206	1.27	0.206	1.25	1.27 ± 0.002	126.59 ± 1.245
6.25	0.258	1.58	0.239	1.45	0.267	1.65	0.241	1.46	1.53 ± 0.007	153.38 ± 4.762
12.5	0.434	2.65	0.433	2.63	0.463	2.86	0.433	2.62	2.69 ± 0.007	268.85 ± 5.794*
25	1.067	6.51	1.057	6.41	1.064	6.57	1.083	6.55	6.51 ± 0.006	650.75 ± 3.592*#
50	1.407	8.58	1.472	8.92	1.237	7.64	1.254	7.58	8.18 ± 0.058	818.06 ± 33.740*#

Table 3: Effect of α-MSH on melanogenesis. B16F10 cells were treated with 0.001, 0.01, 0.1, 1 and 10 μM α-MSH for 72 hours. Melanin content in the treated cells were determined and compared to the untreated control. The results are presented as the mean ± S.E.M. from 2 independent experiment (n=2). * $p < 0.01$, significantly when compared to untreated control. # $p < 0.01$, significantly when compared to α-MSH 0.001 μM. ** $p < 0.01$, significantly when compared to α-MSH 0.01 μM. ## $p < 0.01$, significantly when compared to α-MSH 0.1 μM.

α-MSH (μM)	N1		N2		Average fold of stimulation	%Stimulation of melanin content
	OD 405nm	Fold of stimulation	OD 405nm	Fold of stimulation		
Untreated control	0.164	1	0.165	1	1.0±0.0005	100.00
0.001	0.406	2.47	0.405	2.45	2.46±0.001	245.91±1.20*
0.01	0.509	3.10	0.505	3.05	3.08±0.002	307.58±2.14*#
0.1	0.511	3.11	0.524	3.17	3.14±0.006	313.93±2.99*#
1	0.569	3.46	0.604	3.65	3.56±0.018	355.58±9.68*#**##
10	0.577	3.51	0.608	3.67	3.59±0.015	359.07±8.31*#**##

Table 4: Effect of xanthoxylin on cells viability. B16F10 cells were treated with 3.125, 6.25, 12.5, 25 and 50 µg/ml xanthoxylin for 72 hours. Viability of the treated cells were determined by staining with 1 mg/ml resazurin solution at 37°C for 4 h. The percentage of cell survival was calculated from the absorbance at 570 and 600 nm. The results are presented as the mean ± S.E.M. from 4 independent experiment (n=4). * $p < 0.01$, significantly when compared to untreated control.

Xanthoxylin (µg/ml)	N1		N2		N3		N4		Average of % cell viability
	OD	% Cell viability	OD	% Cell viability	OD	% Cell viability	OD	% Cell viability	
Untreated control	1.463	100.00	1.464	100.00	1.499	100.00	1.467	100.00	100.00±0.009
3.125	1.653	113.02	1.653	112.91	1.675	111.74	1.66	113.16	112.71±0.005*
6.25	1.603	109.60	1.534	104.75	1.561	104.17	1.533	104.50	105.75±0.016
12.5	1.562	106.77	1.525	104.16	1.528	101.94	1.577	107.53	105.10±0.013
25	1.444	98.74	1.448	98.91	1.371	91.46	1.434	97.79	96.72±0.018
50	1.245	85.13	1.298	88.63	1.335	89.06	1.267	86.37	87.30±0.019*

Table 5: Effect of protein kinase inhibitors on xanthoxylin-induced melanogenesis. B16F10 cells were pretreated with 5 μM PKA, PKB, PKC and MEK1 inhibitors at 37°C for 1 hour and then treated with 25 μg/ml xanthoxylin at 37°C for 72 hours. The results are presented as the mean ± S.E.M. from 3 independent experiment (n=3). * $p < 0.01$, significantly when compared to control. # $p < 0.01$, significantly when compared to xanthoxylin 25 μg/ml alone. ** $p < 0.01$, significantly when compared to cells were pretreated with PD98059. ## $p < 0.01$, significantly when compared to cells were pretreated with Ro-32-043.

Treatment	N1		N2		N3		Average of fold of stimulation	%Stimulation of melanin content
	OD 405nm	Fold of stimulation	OD 405nm	Fold of stimulation	OD 405nm	Fold of stimulation		
Untreated control	0.176	1	0.176	1	0.170	1	1±0.002	0
Xanthoxylin 25 μg/ml alone	1.499	8.49	1.438	8.17	1.471	8.63	8.43±0.017	100.00*
Xanthoxylin 25 μg/ml with H-89 5μM	0.619	3.51	0.677	3.85	0.671	7.57	3.77±0.018	44.66±1.73*#
Xanthoxylin 25 μg/ml with Ro-32-043 5μM	0.967	5.48	1.052	5.98	0.947	5.56	5.67±0.032	67.25±2.89*#
Xanthoxylin 25 μg/ml with LY294002 5μM	1.252	7.10	1.212	6.89	1.255	7.36	7.12±0.014	84.40±0.51*#**
Xanthoxylin 25 μg/ml with PD98059 5μM	1.256	7.12	1.255	7.13	1.291	3.94	7.28±0.012	86.28±1.24*#**##

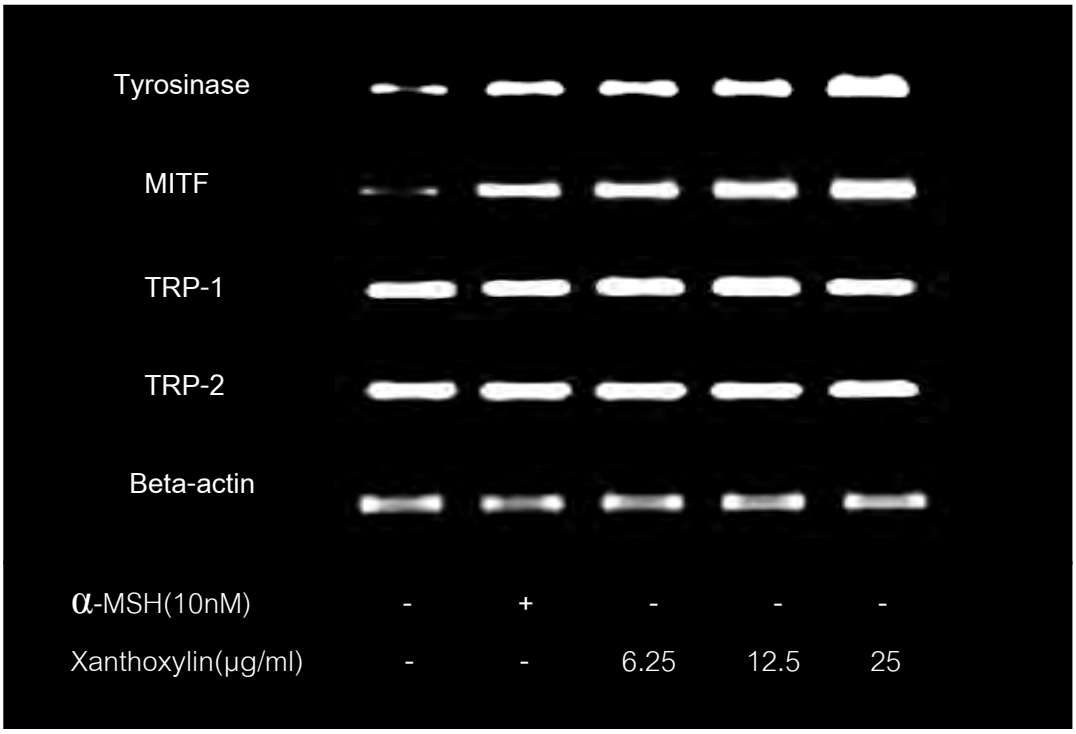

Figure 31: Effect of xanthoxylin on tyrosinase, MITF, TRP-1 and TRP-2 expression (n1). B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 in these cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

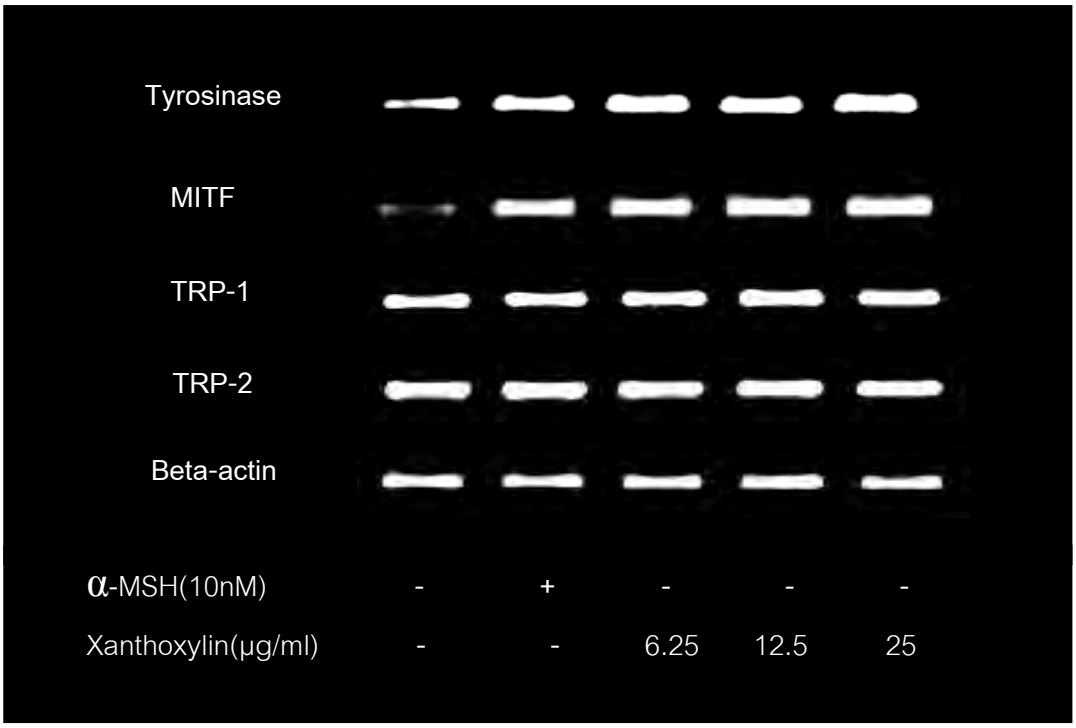

Figure 32: Effect of xanthoxylin on tyrosinase, MITF, TRP-1 and TRP-2 expression (n2). B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 in these cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

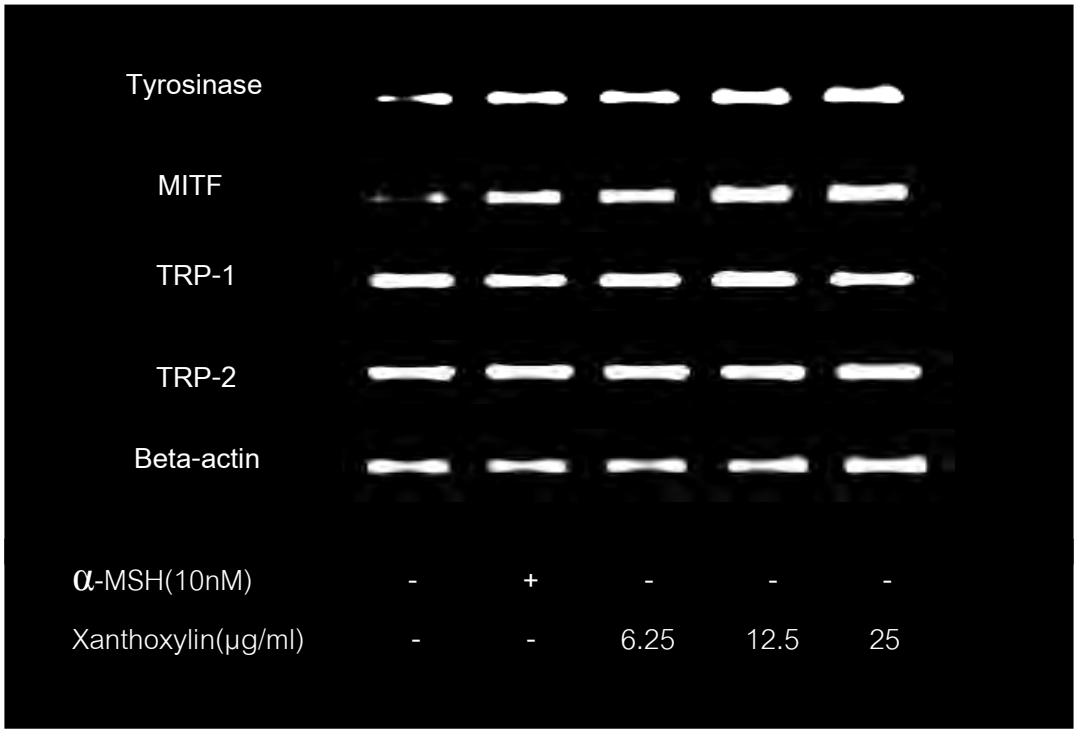

Figure 33: Effect of xanthoxylin on tyrosinase, MITF, TRP-1 and TRP-2 expression (n3). B16F10 cells were treated with 6.25, 12.5 and 25 μg/ml xanthoxylin for 72 hours. Untreated and 10 nM α-MSH-treated cells were used as the negative and positive controls, respectively. The mRNA expression of tyrosinase, MITF, TRP-1 and TRP-2 in these cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

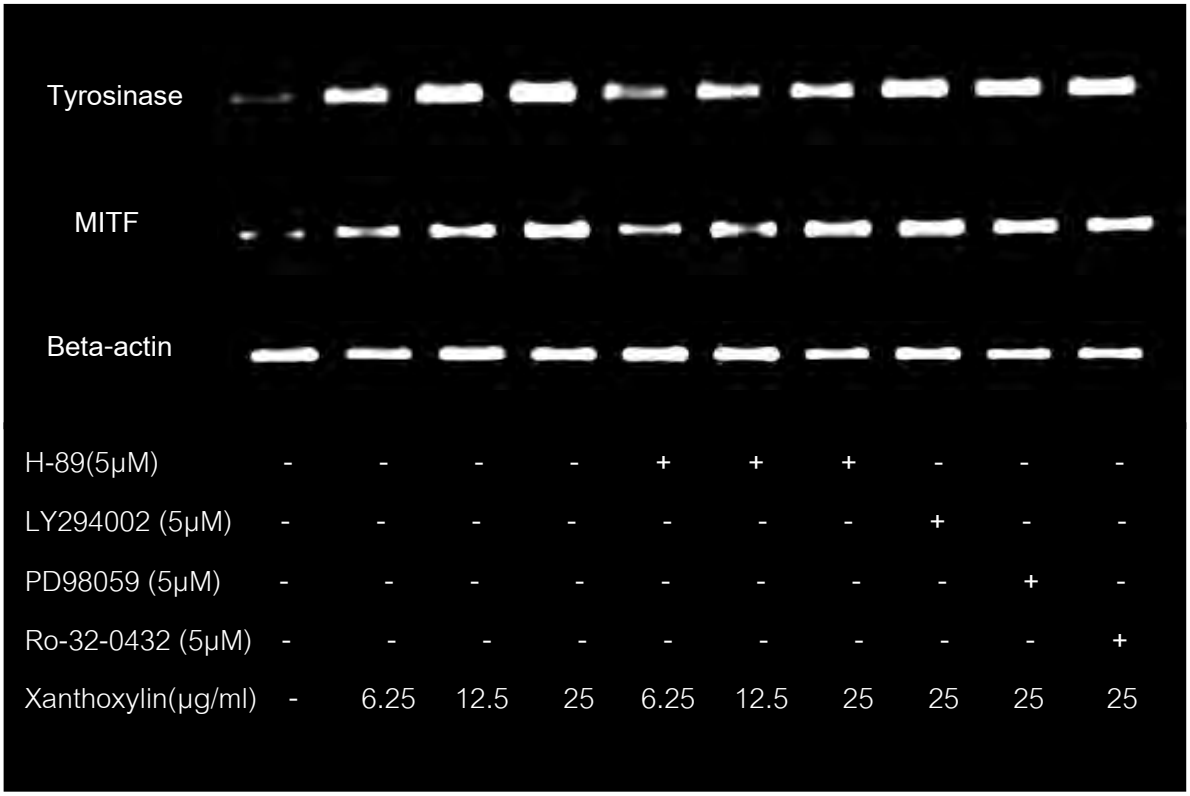

Figure 34: Effect of protein kinase inhibitors on xanthoxylin-induced tyrosinase expression (n1). B16F10 cells were pretreated with 5 μM protein kinase inhibitor for 1 hour and then treated with xanthoxylin for 72 hours. The mRNA expression of tyrosinase and MITF in the treated cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

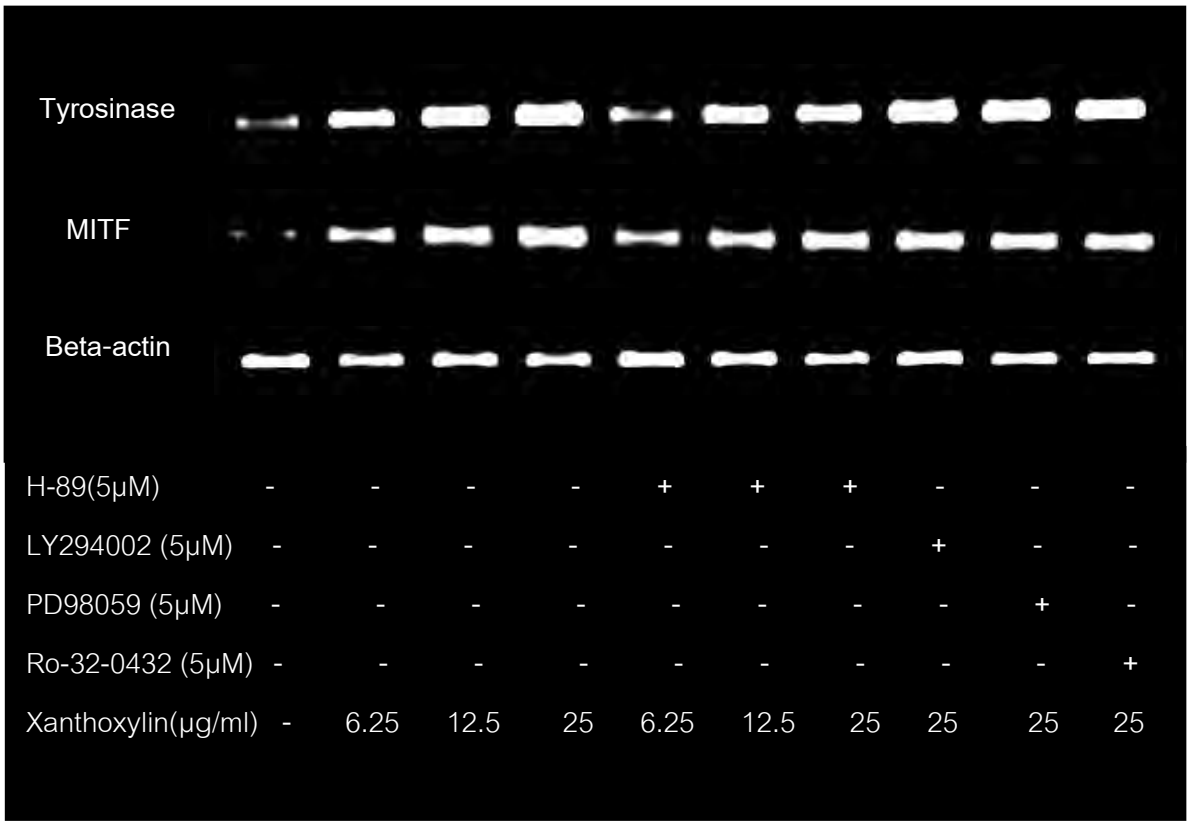

Figure 35: Effect of protein kinase inhibitors on xanthoxylin-induced tyrosinase expression (n2). B16F10 cells were pretreated with 5 μM protein kinase inhibitor for 1 hour and then treated with xanthoxylin for 72 hours. The mRNA expression of tyrosinase and MITF in the treated cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

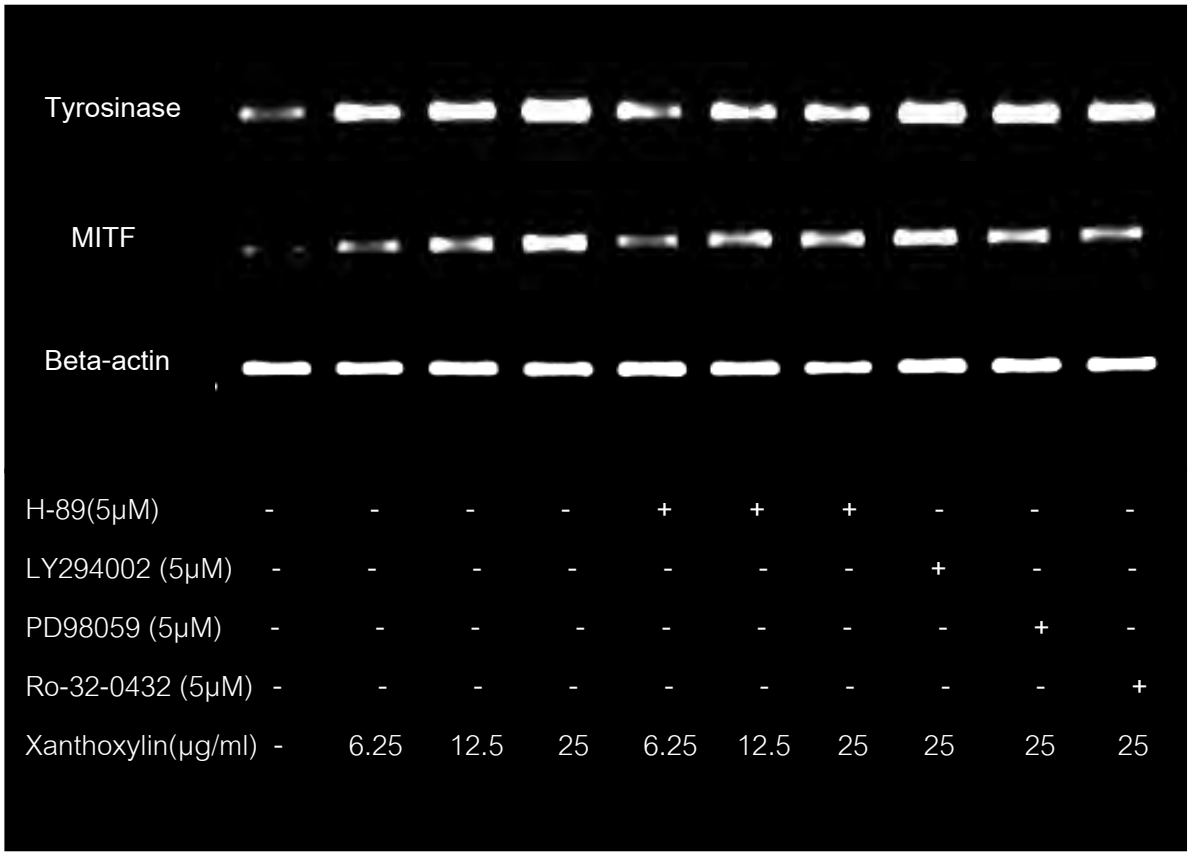

Figure 36: Effect of protein kinase inhibitors on xanthoxylin-induced tyrosinase expression (n3). B16F10 cells were pretreated with 5 μM protein kinase inhibitor for 1 hour and then treated with xanthoxylin for 72 hours. The mRNA expression of tyrosinase and MITF in the treated cells were identified by RT-PCR with specific primers. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation.

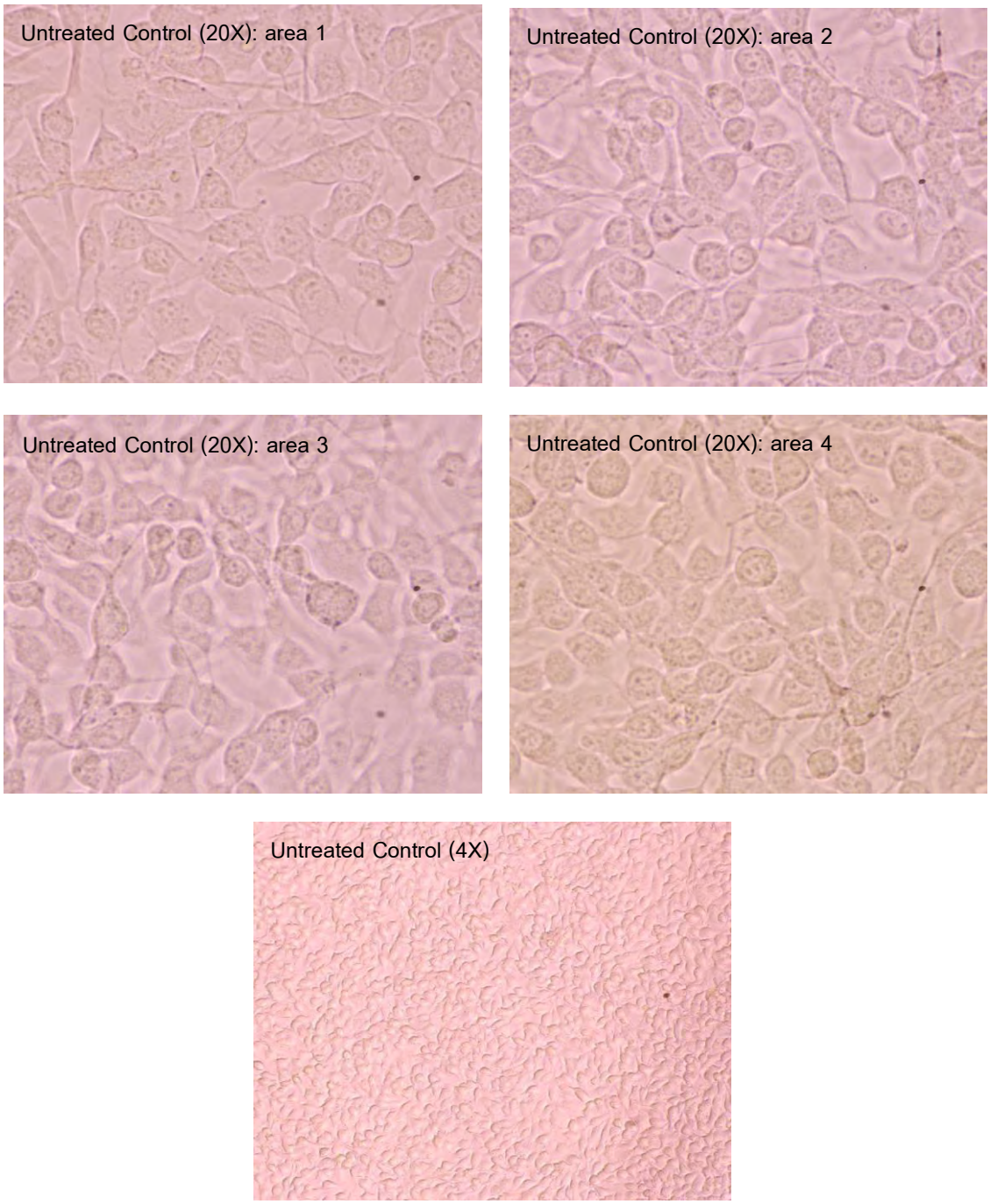

Figure 37: A representative results of untreated control on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiment; n=3).

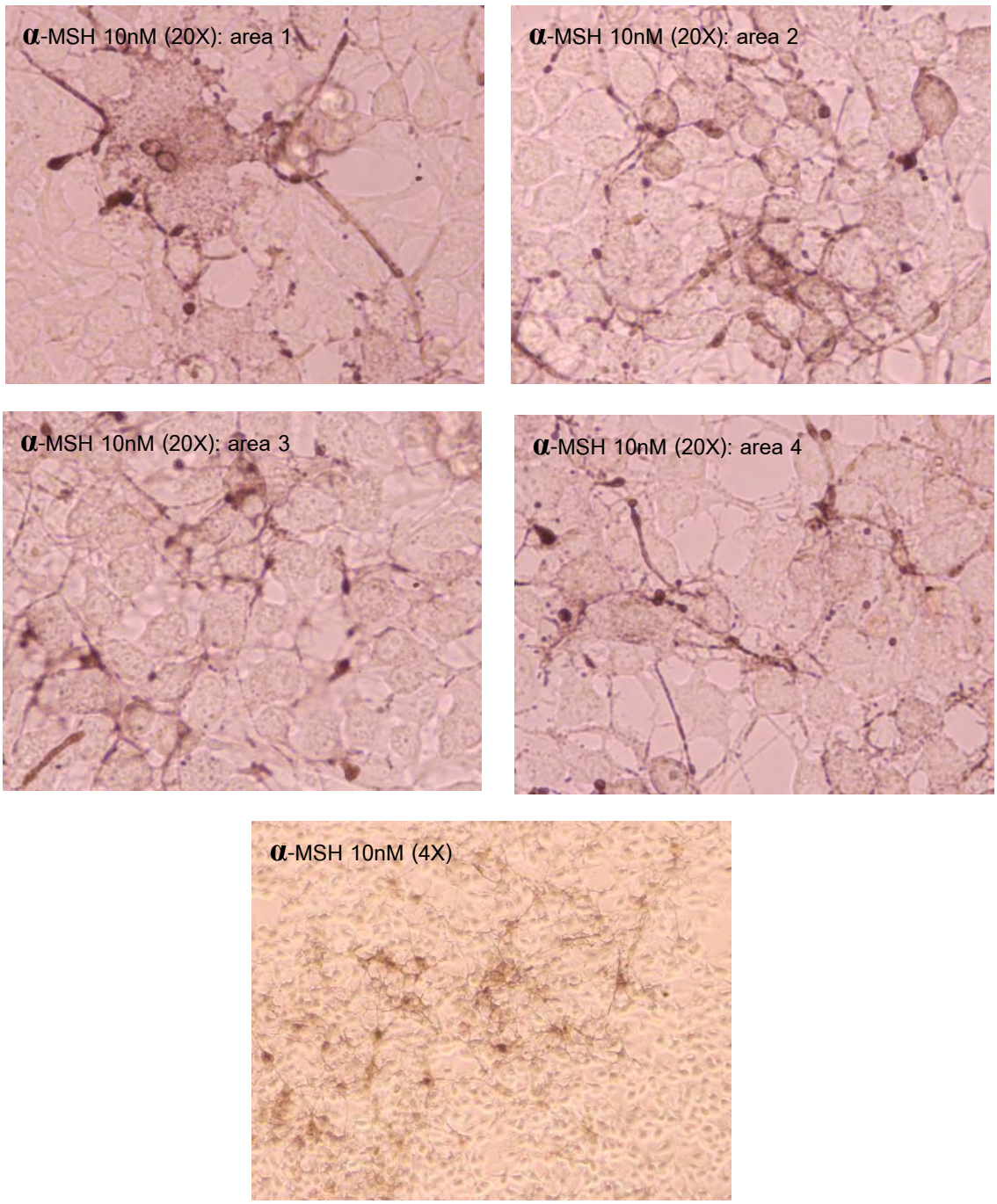

Figure 38: A representative results of α-MSH at 10 nM (positive control) on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiment; n=3).

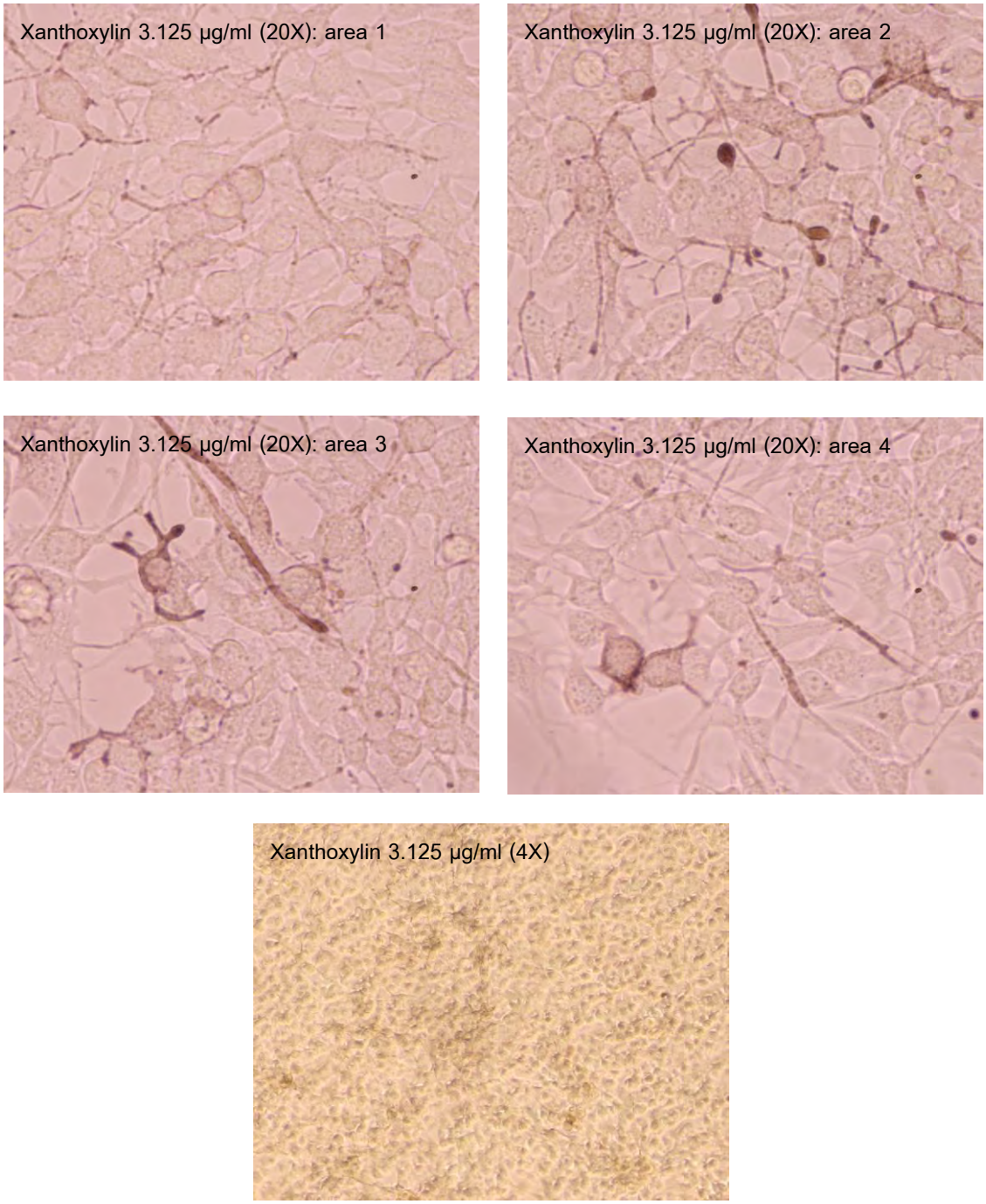

Figure 39: A representative results of the effect of xanthoxylin at 3.125 µg/ml on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiments; n=3).

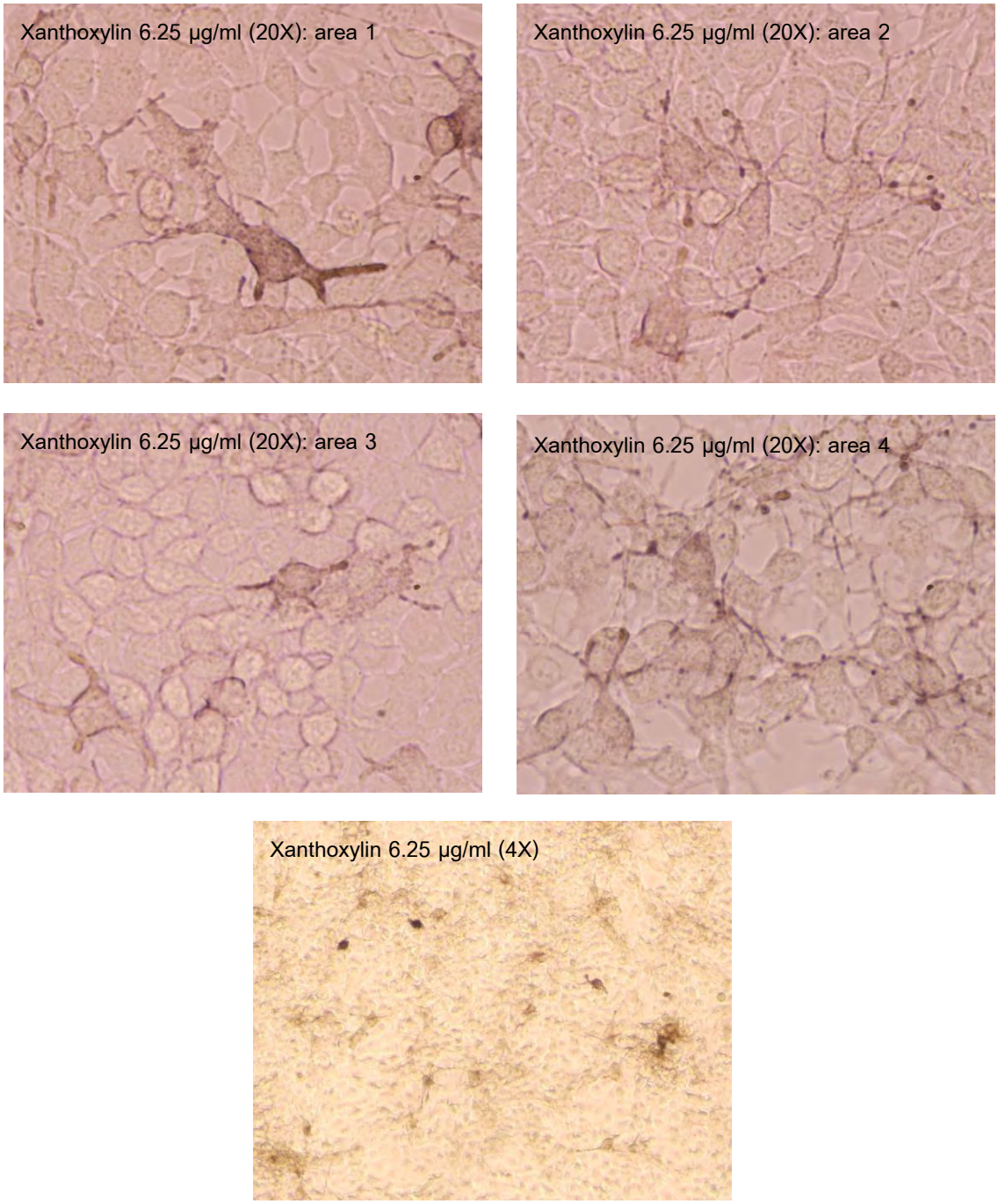

Figure 40: A representative results of the effect of xanthoxylin at 6.25 µg/ml on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiments; n=3).

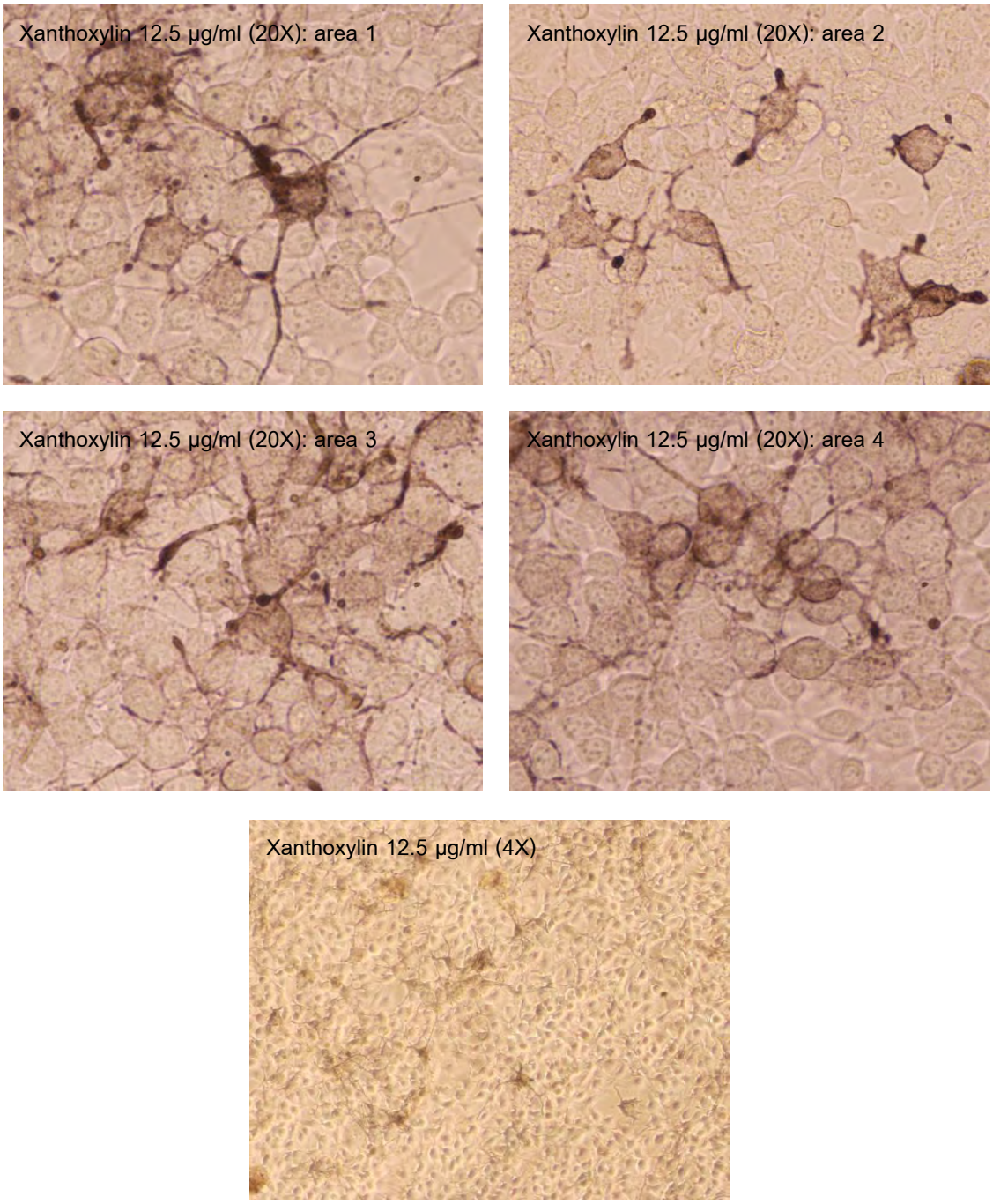

Figure 41: A representative results of the effect of xanthoxylin at 12.5 µg/ml on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiments; n=3).

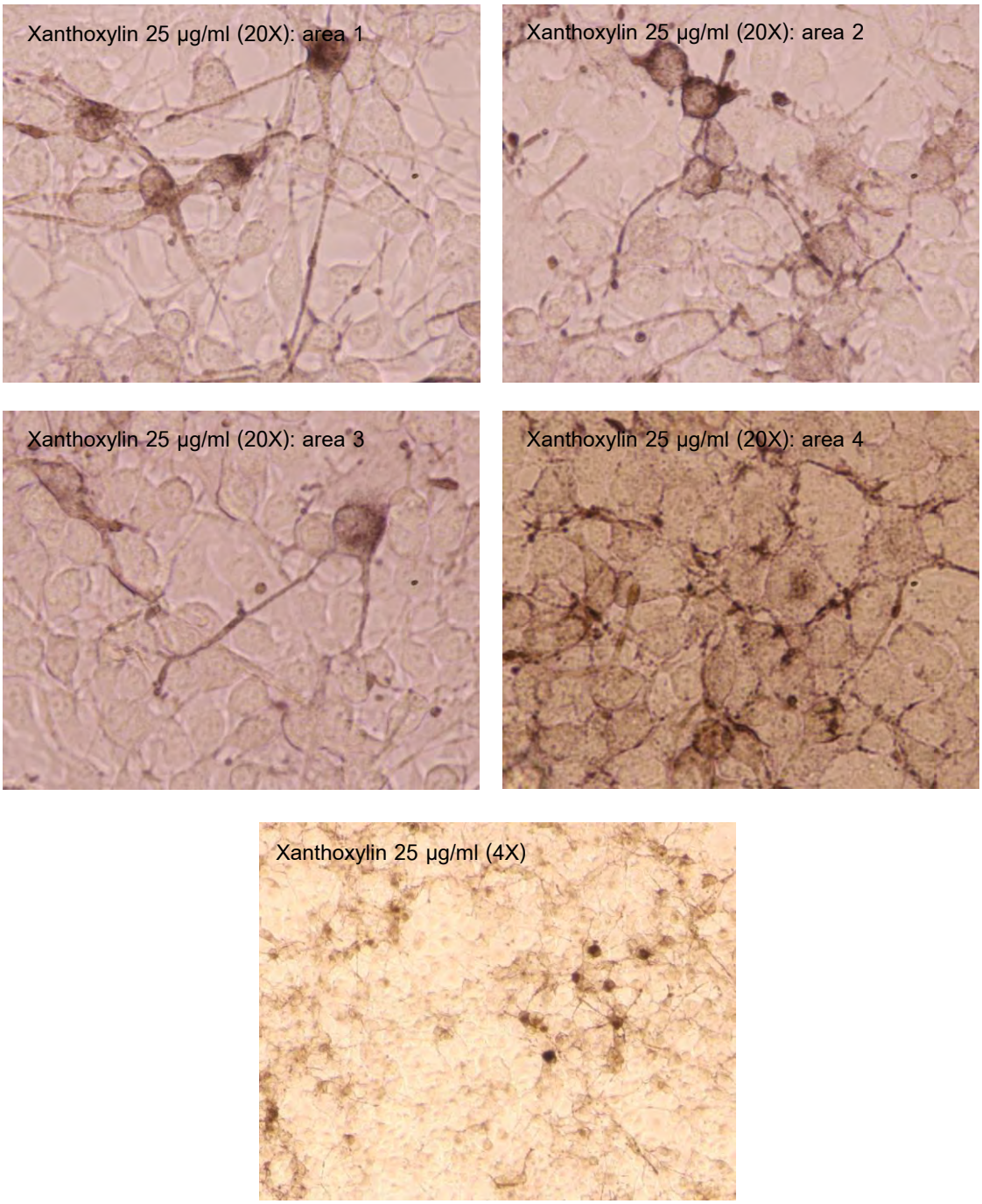

Figure 42: A representative results of the effect of xanthoxylin at 25 µg/ml on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiments; n=3).

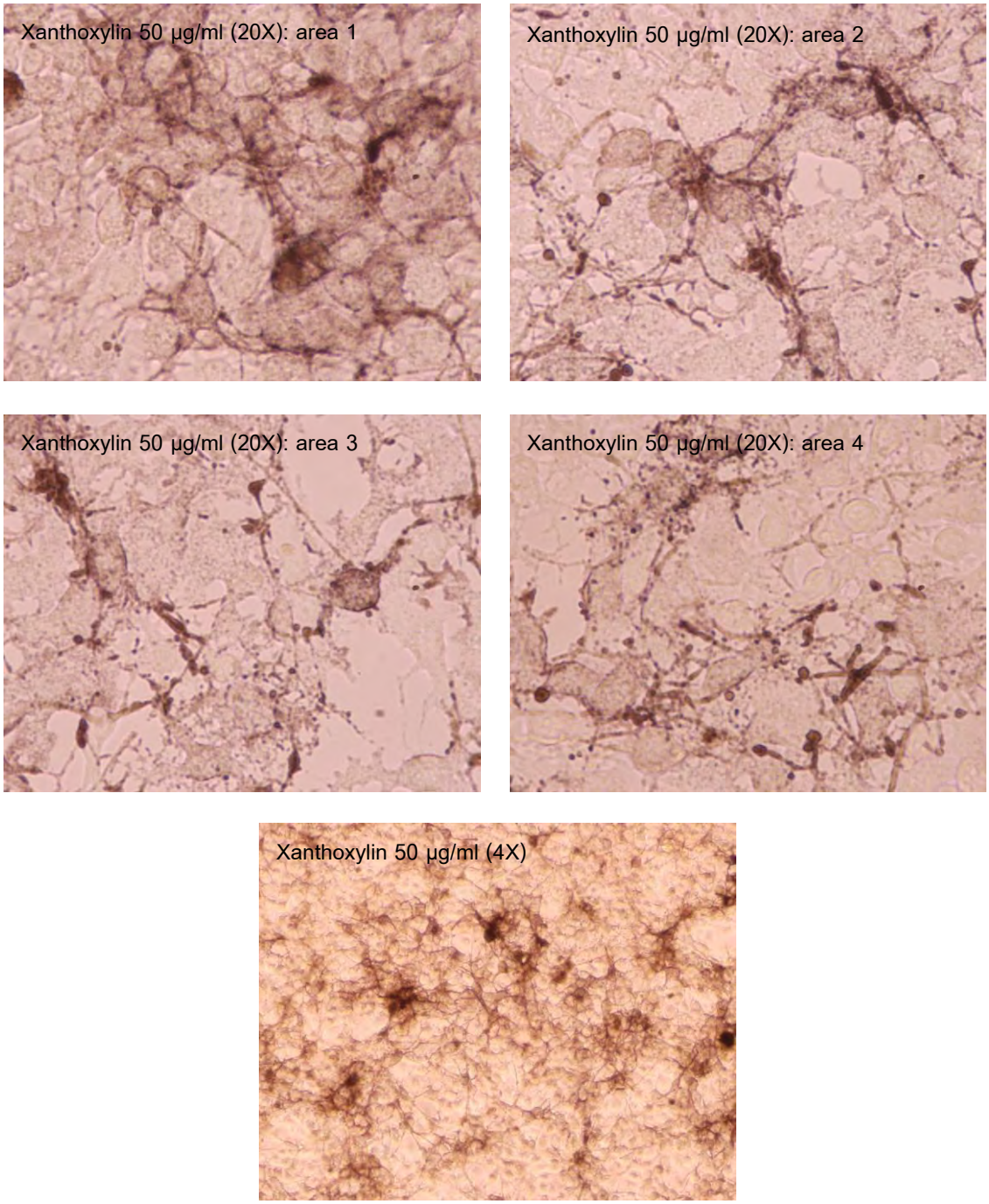

Figure 43: A representative results of the effect of xanthoxylin at 50 µg/ml on dendricity of mouse B16F10 melanoma cells under light microscope 20X have 4 areas and 4X; (from 3 independent experiments; n=3).

BIOGRAPHY

Miss Wanmai Moleephan was born on January 16, 1985 in Ubonratchatani, Thailand. She was graudated Bachelor degree of Science (Biotechnology) in 2007 from Faculty of Industrial Technology, Silpakorn University.

Poster presentation article “EFFECTS OF XANTHOXYLIN ON MELANOGENESIS” of Miss Wanmai Moleephan was presented in Thai Journal of Pharmacology vol.32, No1, 2010, Proceedings of 32st Pharmacological and Therapeutic Society of Thailand Meeting 25-26 March 2010 at Thammasat University.