# CHAPTER I

# INTRODUCTION

## 1. Background and Rationale

Regardless the protective role of melanin against the deleterious effects of ultraviolet radiation, its overproduction can lead to the development of melasma [1-3], which is cosmetically impaired mental health of an affected individual. There are various factors and conditions, in addition to sun light, known to induce melasma. Included in this list is nutrition deficiency, drugs (e.g. anti-epileptic and oral contraceptives), changes in hormone levels during pregnancy and genetic factor.[4] Therefore, understanding the mechanisms involved in melanin synthesis could be therapeutic intervention for melasma treatment.

Normally, melanogenesis occurs in melanocytes, which are dendritic cells lining at the junction of the dermis and epidermis of the skin. The initial rate-limiting step in melanin syntheis is controlled by enzyme tyrosinase. The expression of tyrosinase is regulated by the transcriptional regulator known as microphthalmia-associated transcription factor (MITF). The expression and molecular activity of MITF is known to be regulated by three pathways. The first pathway, leading to an increase in the cytosolic levels of MITF mRNA, is involved the activation of protein Kinase A (PKA) and cAMP response element-binding (CREB) transcription factor. In the second pathway, MITF is phosphorylated through the activation of the Ras/ERK cascade and degraded resulting in transcription inhibition of tyrosinase. In the third pathway, binding of MITF to the tyrosinase promoter is enhanced through the activation of glycogen synthase kinase 3Beta (GSK3β). [5-7]

Although, the formation of melasma can be reduced cosmetically by hydroquinone or glycolic acids found in the skin care products, these active ingredients could induce some side effects, such as an allergic reaction or skin burning. [4, 8-10]

Mounting evidence suggests that the extracts from euphorbiaceous plant could provide an alternative mean to treat melasma.[11, 12] Therefore, in the present study, the crude extracts from two members of Euphorbiaceae family found predominantly in Thailand namely *Mallotus spodocarpus* and *Excoecaria bicolor* have been screened by using mushroom tyrosinase assay and melanocyte cell culture system for tyrosinase inhibitory effect and their cytotoxicity, respectively.

## 2. Research Questions

### Primary Question

Whether the crude extracts from *Mallotus spodocarpus* and *Excoecaria bicolor* possess tyrosinase inhibitory effect and cytotoxicity as determined by mushroom tyrosinase assay and melanocyte-based MTT assay, respectively

### Secondary Question

Whether the crude extracts from *Mallotus spodocarpus* and *Excoecaria bicolor* influence signaling cascades involved in MITF-regulated tyrosinase expression using RT-PCR and western blot analysis

## 3. Research objectives

1. To examine and compare tyrosinase inhibitory effect of the plant extracts using mushroom tyrosinase assay.

2. To examine and compare cytotoxic effect of the plant extracts in cultured of melanocytes using MTT cytotoxic/proliferation assay.

3. To examine the effect of the plant extracts on mRNA expression of tyrosinase and microphthalmia-associated transcription factor by RT-PCR.

4. To examine the effects of the plant extracts on molecular signaling involving in the tyrosinase expression using western blot analysis.

## 4. Hypothesis

The crude extracts from *Mallotus spodocarpus* and *Excoecaria bicolor* could posses tyrosinase inhibitory property and could inhibit tyrosinase expression in cultured melanocytes by interfering with the MITF-associated signaling cascades.

## 5. Keywords

Melanogenesis

Euphorbiaceous plants

*Mallotus spodocarpus*

*Excoecaria bicolor*

## 6. Expected Benefits & Applications

1. To establish melanocyte cell culture model for the screening of plant extracts that could inhibit melanogenesis.

2. To characterize molecular targets of plant extracts in the signaling cascades of melanogenesis.

# CHAPTER II

# REVIEW LITERATURES

## Melanocytes and Skin Melanin

Melanocytes are pigment cells of the skin. They are derived from neural crest cells. During the development, melanoblasts migrate to and situate on the basal layer between the dermis and epidermis. Then, they differentiate and acquire a number of dendritic processes that interdigitate with the surrounding keratinocytes. [13, 14] Mature melanocytes synthesize the melanin pigments through the enzymatic oxidation of the amino acid tyrosine. This process is known as melanogenesis (Figure 1). These melanin pigments protect human skin from UV radiation due to their ability to absorb and reflect UV energy. Melanin pigments also have the ability to scavenge oxidative free radicals, which are thought to play an important role in the initiation and progression of aging. Although melanin is naturally useful, under certain circumstances e.g. hyperpigmentary skin, age spots, post-inflammatory hyperpigmentation and melasma, it is cosmetically considered as a threat .[1, 9]

There are two types of melanin pigments, the black or brown colored eumelanins and the red or yellow colored pheomelanins. The eumelanins are derived from the metabolites of DOPA chrome, whereas the pheomelanins are dereived from metabolites of 5-S-cysteinyl DOPA.[15] Eumelanins are more photoprotective than pheomelanins, with photoprotection increasing in direct proportion to degree of eumelanic skin pigmentation. The switch between eumelanin and pheomelanin synthesis is regulated by a melanocyte-stimulating hormone (MSH), which acts via the second messenger cAMP. Newly synthesized melanin pigments are stored within the cytoplasmic vesicles, the melanosomes, before they are transported along melanocyte dendritic processes to the surrounding keratinocytes, where they are stored.[16, 17]

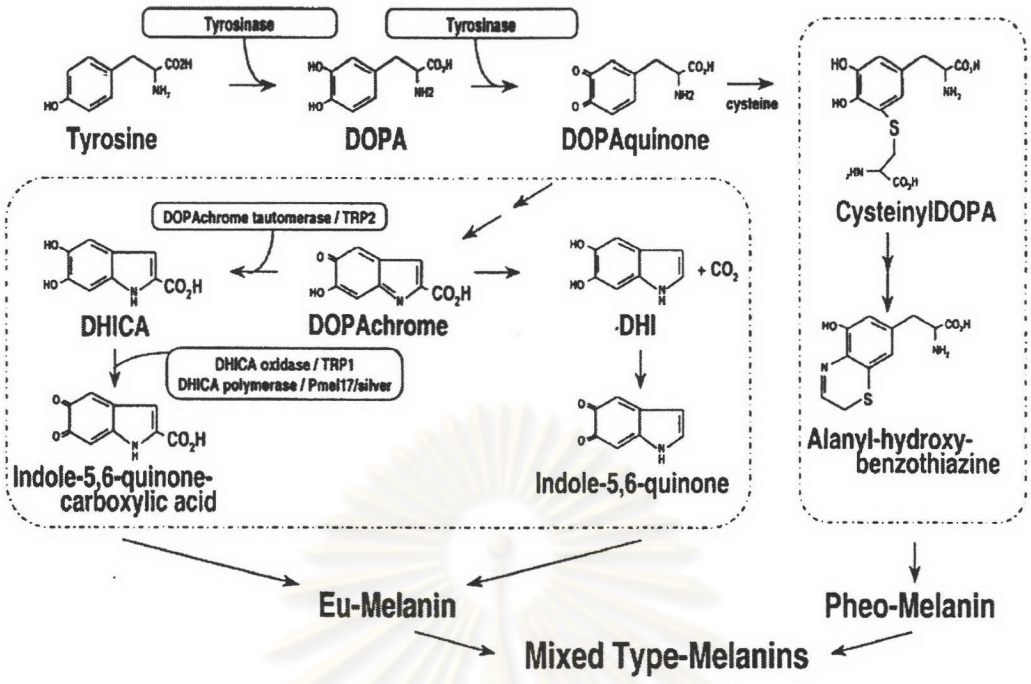


**Figure 1. Melanin biosynthetic pathway (Melanogenesis).** Shown that the Tyrosinase-related protein family is essential for convert tyrosine to two types of melanin. Tyrosinase is the rate-limiting step, whereas TRP-1 and TRP-2 is involved in distal step as indicated in the diagram. [15]

**Melasma** (*"Melas"*, derived from the Greek word, means "black")

Melasma, commonly observed in tropical countries, is an acquired hypermelanosis of sun-exposed areas. It appears as symmetric hyperpigmented macules, which can be confluent or punctuate. The most common locations are on the cheeks, the upper lip, the chin, and the forehead (Figure 2). Melasma has a synonymous term, Chloasma (Greek word *chloazein,* meaning, "To be green.") used to describe the occurrence of melasma during pregnancy. [18] Exposure to sunlight is the major factor causing melasma. UV radiation, particularly UV-A (320-700 nm) can cause peroxidation of lipids in cellular membranes, leading to generation of free radicals, which in turn stimulate melanocytes to produce excess melanin. [9] Hormonal

influences may also play a role in some individuals. The mask of pregnancy is well known to obstetric patients, but the exact mechanism by which pregnancy affects melasma is unknown. It is believed that increased levels of estrogen, progesterone, and melanocyte-stimulating hormone (MSH) during the third trimester of pregnancy may be involved. [18, 19] The treatment of melasma remains topical depigmenting agents. Hydroquinone (HQ) is the most commonly used and has been the gold standard for treatment of hyperpigmentation. It has a hydroxyphenolic stucture that inhibits tyrosinase, leading to the decreased production of melanin. [9, 10, 20] However, HQ could induce some side effects, such as an allergic reaction or skin burning. [4, 8-10]

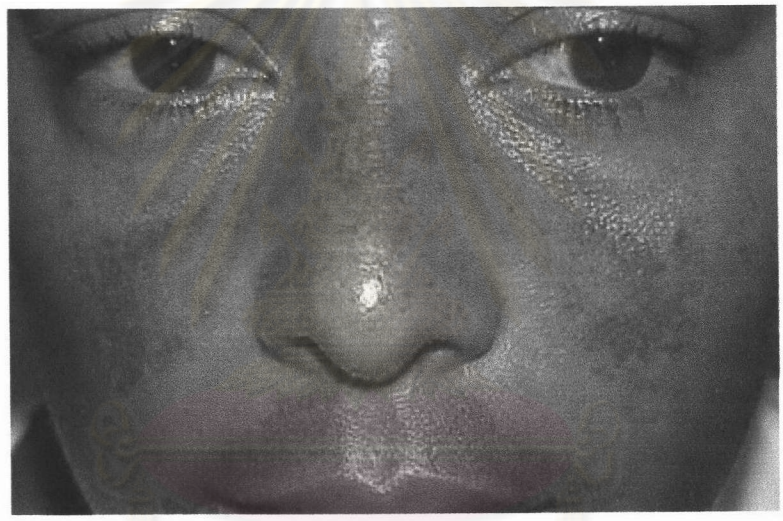

**Figure 2. Characteristics of Melasma.** Melasma appears as brown or gray-brown patches on the face. These patches generally appear on the cheeks, forehead, upper lip, and chin.



**Tyrosinase-related protein family**

Tyrosinase-related protein family (TRPs) is composed of tyrosinase (Tyr), tyrosinase-related protein1 (TRP1) and tyrosinase-related protein2 (TRP2). Tyrosinase (Tyr) is the important rate-limiting enzyme in melanin synthesis (encoded

by the *Tyr/albino* locus). It has a copper monophenol monooxygenases that catalyzes the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinols. This enzyme, found in prokaryotes as well as in eukaryotes, is involved in the formation of pigments such as melanins and other polyphenolic compounds. Tyrosinase binds two copper ions, catalyzes the two initial steps of this process, hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to DOPA quinone and 5,6-dihydroxyindole (DHI) oxidation to indole-5,6-quinone. [14, 18, 21-25]

Tyrosinase-related protein 1 (TRP-1, also known as Tyrp1 and gp75) is expressed specifically in melanocytes and functions in melanin synthesis within melanosomes. It possesses a 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase activity. It is encoded by the *Tyrp1/brown* locus. Tyrosinase-related protein 2 (TRP-2, also is known as Dct and DOPAchrome tautomerase) TRP-2, encoded by the *Dct/slaty* locus, endowed with a DOPAchrome tautomerase activity and producing DHI-2-carboxylic acid (DHICA) from DOPAchrome. TRP-1 and TRP-2 are involved in distal step that control the type of melanin produced. [26-30]

TRPs are encoded by distinct genes on different chromosomes, but they share approximately 40% amino acid identity. TRPs have many similar structural features, including a transmembrane region, two metal-binding regions, and a cysteine rich epidermal growth factor motif, each member of the tyrosinase protein family has a distinct catalytic activity in the biosynthesis of the melanin biopolymer. Tyrosinase, TRP-1 and TRP-2 are localized in the melanosomal membrane and act within the context of a series of reactions in the melanogenic pathway, suggesting an evolutionary relationship. It has been proposed that tyrosinase protein family might interact in a multi-enzyme complex (or metabolon), because each of them contains a cysteine-rich epidermal growth factor motif thought to be involved in protein-protein interactions. [31] Sequencing of TRPs has identified 10-bp sequence (GTCATGTGCT),

which is termed the M-box. The CATGTG motif is recognized by the basic helix-loop-helix/leucine zipper (bHLH-Lz) structure transcription factor known as microphthalmia-associated transcription factor (MITF). MITF is thought to play an important role in regulating the expression of tyrosinase protein family. [32-34, 50]

**Microphthalmia-associated transcription factor**

Microphthalmia-associated transcription factor is a transcription factor that plays a critical role in the differentiation of various cell types, including neural crest-derived melanocytes, mast cells, osteoclasts and optic cup-derived retinal pigment epithelium (RPE). In melanocytes, MITF is essential for melanoblast survival and expression of melanocytic characteristics.[35, 36] It is mapped to chromosome 3p12.3-14.1.[37] Its promoter shares the amino acid similarity with several other transcription factors bearing a bHLH-LZ structure, such as TFE3, TFEB and TFEC, which is transactivated by the transcription factors PAX3, SOX10, and LEF-1.[38-42] MITF exists at least in five isoforms, referred to as MITF-A, MITF-B, MITF-C, MITF-H and MITF-M, differing at their N-termini and expression pattern. The N-terminus of MITF-M (domain M), consists of 11 amino acid residues, is encoded by the melanocyte specific exon 1 (exon1M). Otherwise, the unique N-terminus of MITF-A, MITF-B, MITF-C, or MITF-H is followed by the common region of 83 amino acid residues (domain B1b) (Figure 3). All isoforms with the extended amino-termini share the entire carboxyl portion with MITF-M. MITF-M is exclusively expressed in melanocytes and melanoma cells as a key regulator for melanocyte differentiation. The MITF gene contains multiple promoters, which give rise to unique 5' exons, subsequently spliced on to a common downstream coding region. The expression and activity of MITF is regulated by the cAMP signal transduction pathway. [43-48]

Human MITF can transactivate the tyrosinase promoter primarily through the tyrosinase distal element (positions-1861 to -1842) containing a CATGTG

motif, which is sufficient for directing melanocytespecific transcription of the human tyrosinase gene. Recently, MITF gene has been proposed as a candidate gene responsible for Waardenburg syndrome type 2, which is a dominantly inherited syndrome characterized by sensorineural hearing loss and pigmentary disturbances and Tietz syndrome. [42, 49-56]


**Figure 3. Structures of the Microphthalmia-associated Transcription Factor isoforms.** Shown are the schematic representation of MITF-M and other isoforms, including MITF-A, MITF-B, MITF-C, and MITF-H. All MITF isoforms differ at their N-termini but share the entire carboxyl portion. Domain B1a and domain B1b of MITF-B are encoded by exon 1B. The transcriptional activation domain (AE), the bHLH-LZ structure, and the serine-rich region (S) are indicated. [47]



**Signal Transduction in Melanogenesis**

Exposure to UV radiation can stimulate melanocytes directly increase number of melanocytes in the basal layer of the epidermis, increase size and number of melanosomes, increase production of melanin in melanosomes, increase dendricity of melanocytes, increase transport of melanosomes from melanocytes to keratinocytes, where they are deposited over the nuclei, increase proliferation of keratinocytes, and thickening of the epidermis and stratum corneum.[57] Indirectly, exposure to UV

radiation can stimulate melanocytes through the release of keratinocyte-derived factors such as interleukins, prostaglandins, endothelins, interferons, agouti signal protein and alpha melanocyte-stimulating hormone (α-MSH).[16] α–MSH is the most widely studied physiological agent because it provokes dramatic changes in melanocyte proliferation and morphology, as well as inducing melanin synthesis. α–MSH binds to a melanocortin receptor (MC1R) present on melanocytes and activates, via a G-protein coupled mechanism, the cAMP signal transduction pathway, resulting in the stimulation of melanogenesis by increasing the tyrosinase activity and gene expression.[58] Therefore, pharmacological cAMP-elevating agents such as forskolin, cholera toxin, dibutyryl cAMP, theophylline and isobutyl-methylxanthine can mimic the melanogenic effects of α-MSH. [16]

Increase in cAMP levels will activate at least three pathways. In the first signaling pathway, cAMP activates the PKA and CREB transcription factor leading to an increase in the expression of MITF. As a result, MITF binds to and activates the tyrosinase promoter to stimulate melanogenesis (Figure 4). The second signaling pathway involves the activation of the Ras/ERK cascade. Activated ERK and RSK (ribosomal S6 kinase) phosphorylate MITF and promote its degradation leading to trancriptional inhibition of tyrosinase expression and melanogenesis. This pathway has been thought to be a feedback mechanism preventing an excessive production of melanin. In the last pathway, cAMP will inhibit phosphatidylinositol 3-kinase (PI3K) and AKT and promotes an activation of GSK3β. GSK3β by phosphorylating MITF on serine 298 increases its binding to the tyrosinase promotor, leading to stimulation of tyrosinase expression. [5, 6, 47]

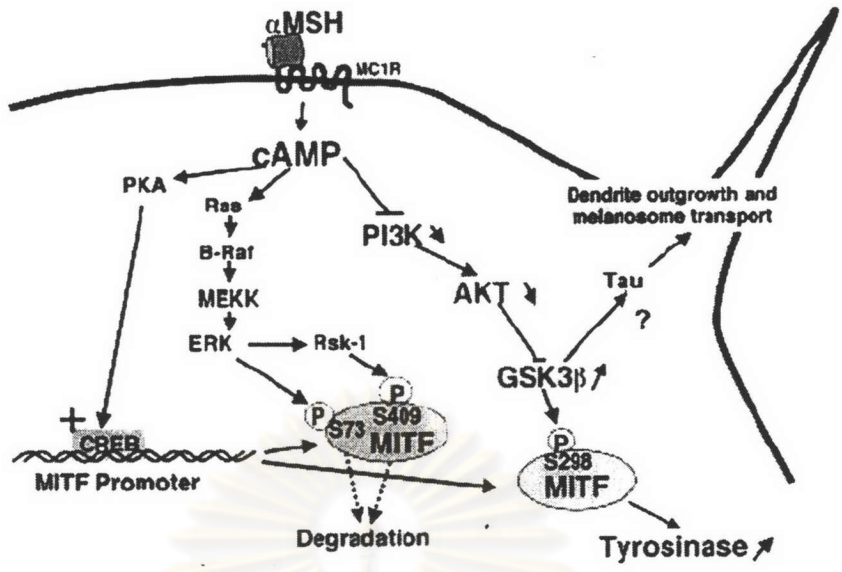


**Figure 4. Model of signaling pathways involved in cAMP-induced melanogenesis.** Elevation of intracellular cAMP content leads to PKA activation and stimulation of MITF transcription resulting in stimulation of tyrosinase expression. cAMP, independently of PKA, also activates the Ras, B-Raf, MEKK, ERK, and Rsk-1 cascade. Phosphorylation of MITF on serine 73 and serine 409 by ERK and Rsk-1, respectively, promotes its degradation constituting a retrocontrol mechanism that prevents an excessive production of melanin synthesis. Finally, cAMP, via a PKA-independent mechanism, inhibits PI3K and AKT and promotes an activation of GSK3β. GSK3β, by phosphorylating MITF on serine 298, increases its binding to the M-box of the tyrosinase promoter, leading to stimulation of tyrosinase expression. [5]

**Botanical aspects of Euphorbiaceae family**

The Euphorbiaceae are mostly monoecious herbs, shrubs, and trees, sometimes succulent and cactus-like, comprising one of the largest families of plants with 300 genera and 7,500 species that are further characterized by the frequent occurrence of milky sap. [59] In ancient Thai medicine, the plants in this family had

been used as antidote, anticancer, and anthelmentic agent. [60] Moreover, recent reports have suggested Euphorbiaceous plants have a diverse range of application (See Table 1)

**Table 1. Euphorbiaceous plants and their diverse range of application**. This table shows the scientific name, the distribution of the plants, parts of plants that have been used for research on their pharmacological actions.

| Scientific name | Distribution | Used | Actions | Ref. |
|---|---|---|---|---|
| *Jatropha curcas* | Tropical | Unknown | Anti-metastasis | 61 |
| *Bridelia ferruginea* | Tropical | Bark | Antimicrobial | 62 |
| *Trewia polycarpa* | India | Root[63] | Anti-inflammatory | 63-69 |
| *Alchornaea cordifolia* | Africa | Leaf[64] | | |
| *Mallotus Spodocapus* | Thailand | Shaw[65] | | |
| *Mallotus oppsitifolium* | Africa | Leaf,Root[66] | | |
| *Croton celtidifolius* | Brazil | Bark[67] | | |
| *Euphorbia royleana* | Tropical | Unknown[68] | | |
| *Croton cajucara* | Amazon | Bark[69] | | |
| *Mallotus philippinensis* | Asia | Fruit | Anti-allergenic | 70 |
| *Jatropha multifida* | India | Root | Antibacterial | 71 |
| *Sapium indicum* | Africa | Fruit | Antimycobacterial | 72 |
| *Euphorbia lathyris L.* | Asia | Seed | Tyrosinase Inhibitory activity | 73 |

*Mallotus spodocarpus* is in Euphorbiaceae family. It distributes in Thailand and Vietnam. It is found in ecological system that have along sunny waysides, in mixed forest, deciduous forest, bamboo forest; soil reported a few times as limestone. It can be called in native names such as; Phaya rak dieo (Eastern); Ta khe khum wang (Phetchaburi); Tao tua mia (Chai Nat). [74]

*Excoecaria bicolor* (Hasshk) is also in Euphorbiaceae family. It has two varieties. It distributes in Thailand, Myanmar, Malaysia, Peninsula, S.China, Indochina and perhaps Taiwan. It can be grown in ecological system that is the evergreen forest, mixed or deciduous forest or scrub, secondary forest, often by streams; scattered to locally common; soil: sandstone. Altitude: sea level to 1520 m. It can be called in alter names such as; Tatum kai Tatum nok, (Prachuap Khiri Khan); Krabue chet tua, Lin krabue khao (Central, Chanthaburi). Its leaflets can be used in pregnant women and used in detoxify the poisonous effect. [74, 75]


**Figure 5. Botanical picture of *Mallotus Spp.* (Left) and *Excoecaria bicolor* (Right)**