CHAPTER II
LITERATURE REVIEW

Skin whitening agents have been widely used to either lighten skin (individuals who wish to change or modify their skin color) or depigment skin (treatment for abnormal skin hyperpigmentation such as melasma, freckles and actinic lentigines). There are several routes by which melanogenesis can be slowed down. Perhaps the best way is to reduce the amount of UV light that reaches the skin. If people cannot stay out of the sun, they should attempt to regularly use sunscreens. UV radiation stimulates the generation of tyrosinase and thus pigmentation. There are several ways to reduce the development of adaptive pigmentation. The most talked about means of reducing pigmentation is the reduction of the tyrosinase activity. This can be accomplished by blocking the production of tyrosinase, inhibiting the enzymatic activity of tyrosinase, or preventing the uptake of tyrosinase by the melanosomes (Farmeco, 1998).

Melanin pigments in skin play a key role in determining skin color and are synthesized by large dendrite cells known as melanocytes, which are located at the epidermal-dermal junction. Tyrosinase in melanocytes is a key enzyme in the synthesis of melanin pigments (Maeda and Fukuda, 1991).

Due to the growing global demand for skin whitening products, it is apparent that not only the efficiency of a whitening formulation but also other factors, such as safety and mildness, are of crucial consideration. Moreover, the cost of the ingredients and formulations plays an increasingly important role (Zuidhoff and Rijsbergen, 2001). Hydroquinone can be quite irritating and cause redness and burning sensation for some individuals. More importantly, it has been shown to cause exogenous ochronosis (a serious cytotoxic side effect) and cytotoxic effect on melanocytes thus is highly toxic to the skin (Hemsworth 1973; Farmeco, 1998). Natural compounds show very promising and better alternative as a skin whitening ingredient since they have relatively fewer side effects (Lee et al., 1997; Kim and Lee, 1998). Several natural compounds being reported in the literature are as follows: Kojic acid, azelaic acid, the plant extracts of

Chaenomeles speciosa, *Dryopteris crassirhizoma*, *Gastrodia ellata*, *Glycyrrhiza glabra* (licorice extract), *Morus alba*, *Myristica fragrans*, *Rheum palmatum*, *Sophora japonica*, *Areca catechu*, *Broussonetia kazinoki* (paper mulberry), *Rheum officinale*, *Uvae ursi* (arbutin), *Artocarpus incisus*, *A. gomezianus* (Haadnun) and *A. lakoocha* (Mahaad). All of the above natural compounds have been shown *in vitro* to have a mushroom tyrosinase inhibitory activity (Matsuda et al., 1994; Iida et al., 1995; Lee and Kim, 1995; Masuda et al., 1996; Lee et al., 1997; Jang et al., 1997; Lee and Choi, 1999; Lee, Kang and Han, 1997; Kim and Lee, 1998; Likhitwitayaruid et al., 2000). Furthermore, such extracts as licorice extract, *Morus alba* extract, paper mulberry and *A. incisus* have already been tested *in vivo* to confirm the compound 's suitability for use in skin whitening products (Jang et al., 1997; Kim and Lee, 1998; Shimizu et al.,1998; Zhai and Maibach, 2001; Zuidhoff and Rijsbergen, 2001).

Artocarpus lakoocha is an interesting compound, which has been found in the north and northeast of Thailand. Puag-Haad is an aqueous extract of the heartwood of *Artocarpus lakoocha*. It has been reported that the main component is 2,4,3′,5′-tetrahydroxystilbene (Mongkolsuk et al., 1957). Yodhabandu (1960) and Poopyruchpong et al. (1978) found 2,4,3′,5′-tetrahydroxystilbene in 51 and 70 percent yield of Puag-Haad. Recently, Sritularak et al. (1998) reported a potent inhibitory effect of the methanolic extract of *A. lakoocha* on enzyme mushroom tyrosinase *in vitro* using L-DOPA as a substrate. Further comparison of its active constituent, 2,4,3′,5′-tetrahydroxystilbene (oxyresveratrol), showed that the compound had a concentration exhibited of 50% inhibition (IC_{50}) about 1.5 μM, which was 17.9 times higher than kojic acid and 12.9 times higher than norartocarpetin, the active component of *A. gomezianus* (Haadnun) root extract (Sritularak, 1998; Likhitwitayawuid et al., 2000). The IC_{50} value for 2,4,3′,5′-tetrahydroxystilbene was in agreement with Shin et al. (1998) and Kim et al. (2002), who reported the value of 1.0 and 1.2 μM, respectively.

Tiptabainkarn (1967) found that 2,4,3′,5′-tetrahydroxystilbene (Figure 1), the active compound of Puag-Haad (phenolic stilbene), possessed oxidation tendency. To achieve the Puag-Haad stability, we can simply protect it from light or additive

antioxidants. Therefore, the possibility to develop formulation of Puag-Haad as a new whitening cosmetic product is very high.

Colors and skin colors

Melanocytes are components of the melanin pigmentary system, which when fully developed is made up of melanocytes distributed in various sites: the eye (retinal pigment epithelium, uveal tract); the ear (in the stria vascularis); the central nervous system (in the leptomeninges); the mucous membranes; the hair (in the hair matrix); and in the skin (at the dermal-epidermal interface) and occasionally in the dermis. In the skin, melanocytes are located in the basal layer and they project their dendrites into the malpighian layer of the epidermis where they transfer melanosomes to keratinocytes (Fitzpatrick et al., 1993).

Melanosomes are highly organized ellipsoidal organelles that contain melanin inside a unit membrane and deposit it on an internal filamentous and/or microvesicular matrix. In melanosomes of normal skin, melanin is an extremely dense, virtually insoluble polymer of high molecular weight and is always attached to the structural protein. In theory, mammalian melanin pigments have one of two chemical compositions: (1) eumelanin, a brown polymer derived from the conversion of the amino acid, tyrosine, to an alkali-insoluble brown/black chromophore; or (2) pheomelanin, a yellow –reddish, alkali-soluble pigment derived from tyrosinase. However, which one of the intermediates in the tyrosine-melanin pathway (DOPA-DOPAquinone) combines with cysteine (or glutathione) to form cysteinyl DOPA. This leads to the yellow pigment, pheomelanin. The conversion of tyrosine to DOPA and DOPA to DOPAquinone is accomplished by an aerobic copper-containing oxidase, tyrosinase (Figure 2). Many melanins appear to be mixtures of pheomelanic and eumelanic monomers. The ratio of the monomers varies among different samples of melanin (Riley, 1977; Prota, 1996).

Each epidermal melanocyte secretes melanosomes into a finite number of neighboring keratinocytes (approximately 36): this partnership of a melanocyte and a neighboring group of keratinocytes is called an epidermal melanin unit (Figure 2). After melanosomes have been transferred to keratinocytes of the epidermal, they are

surrounded by a membrane, becoming secondary lysosomes. In white skin, the melanosomes are relatively small and form groups within the secondary lysosomes. This is now called a melanosome complex.

Melanin pigmentation of human skin is divided into two components: constitutive and facultative skin colors (Fitzpatrick et al., 1993; Tabibian, 2000). Constitutive skin color is the amount of cutaneous melanin pigmentation generated according to cellular genetic programs, without any direct effect by radiation of solar origin. It is the level of pigmentation acquired in those parts of the body habitually shielded from light. Facultative (inducible) skin color or "tan" includes the short-lived immediate tanning (IT) and delayed tanning (DT) reactions, both of which are generally elicited by direct exposure of the skin to UVR (Table 1). Because hyperpigmentation of the skin tends to be discontinued, facultative color change is reversible. The hyperpigmentation induced by endocrine changes during pregnancy and Addison's disease is another type of facultative color change. Alterations in endocrine balance can significantly affect the response of human skin to UVR. Facultative color changes in humans, then, arise from the complex relationship between light, hormones and the genetic potential of the epidermal melanin unit, the basic multicellular "organ" of melanin metabolism (Figure 3).

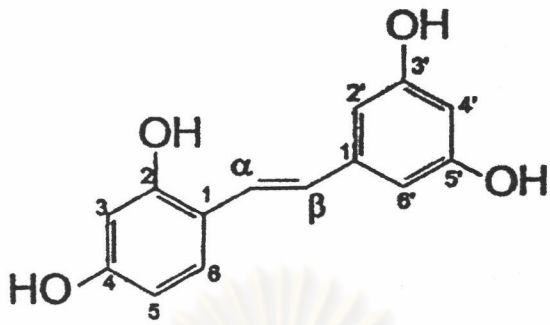

Figure 1 Chemical structural of 2, 4, 3′, 5′- tetrahydroxystilbene (oxyresveratrol)

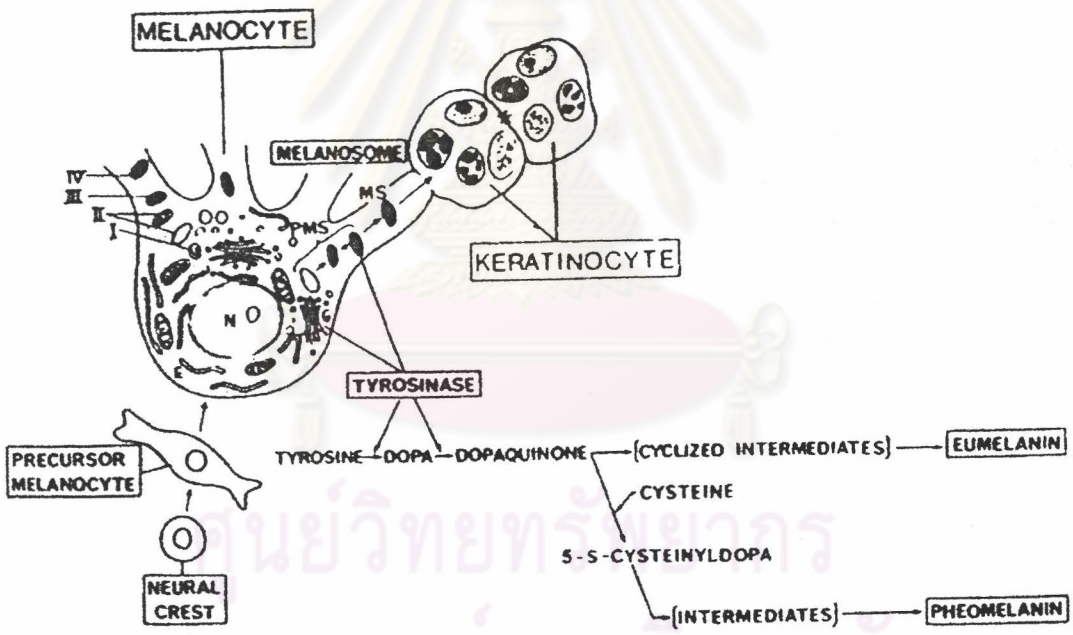

Figure 2 The epidermal melanin unit. Diagram summarizing major in the early development of melanocyte, their subsequent differentiation, and their interaction throughout life.

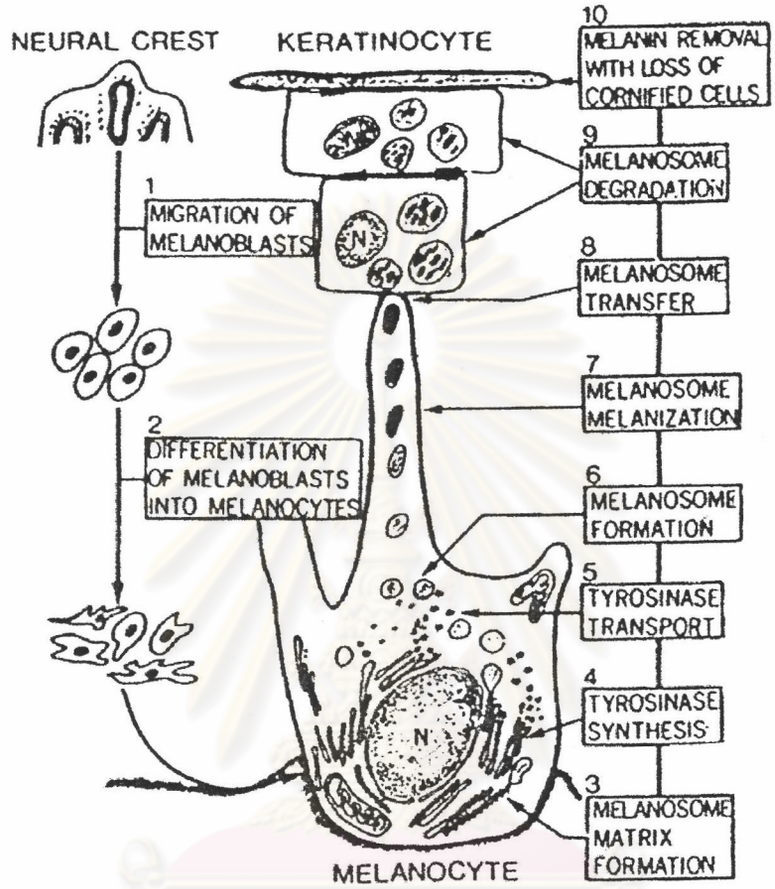

Figure 3 Morphologic and metabolic pathway of epidural melanin presentation. Also shown is migration of melanocyte precursors (melanoblasts) from the neural crest to the skin and differentiation of melanocytes within the epidermis.

Table 1 The responses of melanocytes and the induction of melanogenesis after exposure to sunlight

Melanocyte response and melanogenesis	Immediate tan (UVA and visible light)	Delayed tan (UVA and UVB)
Sunlight	UVA (320-400 nm), much less UVB	UVB (290-320 nm), less by UVA (320-400 nm)
Onset	Instantaneous, during exposure; Fades immediately	Gradual, beginning at 48 to 72 h; lasts for weeks
Melanogenesis		
Melanocyte	No numerical increase	Numerical increase by multiple exposures
Melanin	Photooxidation of preformed melanin	Synthesis of new melanin
Tyrosinase	No increase activity	Markedly increased activity
Melanosome	No new synthesis	New synthesis; increase in transfer to keratinocytes

The types of skin according to Fitzpatrick classification (Fitzpatrick, 1993) are shown in table 2. Zuidhoff and Rijsbergen (2001) suggested that we should measure melanin content using the Mexameter MX 16 for skin type classification. The Mexameter is designed to measure skin color specifically and measures the erythema and melanin content by a light reflection method. The scale range from 400 to 700. Range indications:

I Celtic type (very fair skin, red hairs, freckles), average readings (for melanin only): 400 – 470

II Caucasian white (fair skin, blond, blue eyes): 410 – 490

III Mixed type (blond-brown hair, brown eyes, little bit darker skin): 420 – 510

IV Mediteranean type (dark hair, darker skin, brown eyes): 420 – 520

V Dark skin: 450 – 550

VI Black skin: 520 – 700

Table 2 Skin types I – VI according to Fitzpatrick classification

Skin type	Sunburn and Tanning History*	Example
I	Always burns easily; never tans	People most often with fair skin, blue eyes. Freckles; unexposed skin is white
II	Always burns easily; tans minimally	People most often with fair skin, red or blond hair, blue hazel, or even brown eyes; unexposed skin is white
III	Burns moderately; tans gradually and uniformly (light brown)	Normal average white person; unexposed skin is white
VI	Burns minimally; always tans well (moderated brown)	People with white or light brown skin, dark brown hair, dark eyes (e.g., Mediterraneans, Mongoloids, Orientals, Hispanics); unexposed skin is white or light brown
V	Rarely burns, tan profusely (dark brown)	Browns-skinned person (e.g., Amerindians East Indians, Hispanics)
VI	Never burns; deeply pigmented (black)	Blacks (e.g., African and American Negros, Australian and South Indian Aborigines); unexposed skin is black

* Based on first 30 to go minutes' sun exposure after winter season or no sun exposure

Biosynthesis and regulating factors in melanin pigmentation

Pigmentation is regulated by complex processes ranging from the molecules, which are relevant to melanin synthesis, to symbiotic interactions with keratinocytes of the skin as a fully integrated system (Fitzpatrick et al., 1993). The key events in these processes include the following (Figure 4):

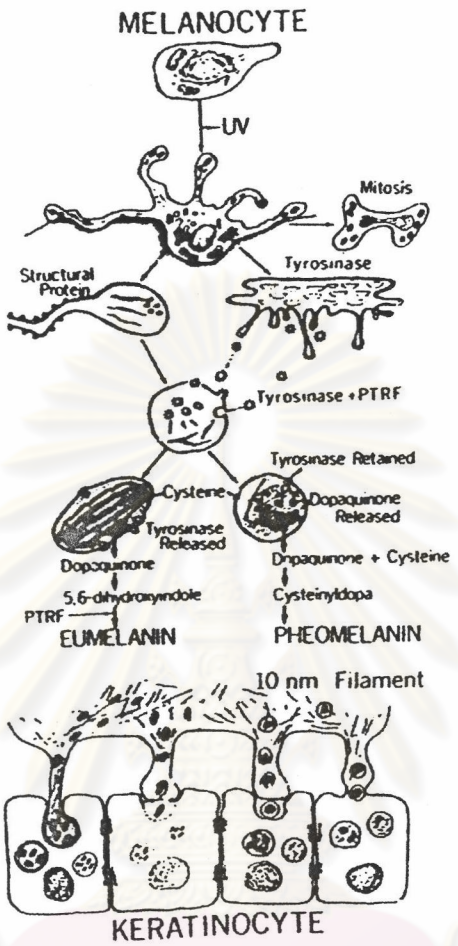

Figure 4 A cascade of melanogenesis after exposure to UVR.

1. Migration of the melanoblasts from the neural crest.
2. Differentiation of the melanoblasts to form epidermal melanocytes.
3. Formation of structural proteins in stage I melanosomes.
4. Formation of the enzyme tyrosinase in the Golgi complex.
5. Transport of tyrosinase from the Golgi complex to stage I melanosomes.
6. Assembly of tyrosinase and structural protein in the formation of stage II melanosomes within melanocytes.
7. Melanization of melanosomes to form stage III-IV melanosomes.
8. Movement of stage IV melanosomes from the perikaryon to the dendritic processes of the melanocyte and their transfer to and incorporation within the keratinocytes, either as single, discrete particles or as complex, aggregated particles (i.e., "melanosome complexs")
9. Degradation of melanosomes within the keratinocytes.
10. Removal of melanin/melanosomes within along with loss of stratum corneum.

The above processes for formation of melanosomes and their melanization may be viewed as a "cascade" of events, channeled by internal regulatory controls that emerge as the melanosomes become programmed to pursue their fate. This process may be outlined in the following series of events (Figure 3).

Tyrosinase

The enzyme responsible for the oxidation involved in melanogenesis is tyrosinase. At least two forms (α and β) of the enzyme are recognized and minor differences in activity exist but the general properties are broadly identical. They are widespread in nature, occurring in both eukaryotic and prokaryotic organism. In vertebrates the enzyme is synthesized only in specialized cells (melanocytes) and is active only in specialized cytoplasmic organelles (melanosemes) (Riley, 1977; Nichita et al., 1999).

Tyrosinase is rate limiting, essential enzyme in the biosynthesis of the skin pigment melanin (Ferrer et al., 1995). It converts tyrosine to DOPA, and then DOPAquinone, which is cyclized and further oxidized to give rise to eumelanin. If

DOPAquinone encounters cysteine, pheomelanin is formed via cysteinylDOPA (Figure 5) (Fitzpatrick et al., 1993). The rate limiting steps in melanogenesis are the oxidation of tyrosine and DOPA. The quantity of melanin synthesized is thus proportional to the amount of tyrosinase activity present in the cell.

The action mechanism of the whitening agents

The following types of whitening ingredients can be described, each having a different mechanism of action and as a result of that, interfering in different ways in the process of tanning (Prota, 1996; Zuidhoff, 2001).

1. Cut off UV: Sunscreen agents (no effect on pigmentation except blocking UV light)
2. Scanvenges free radicals: Tocopherol (less whitening effect)
3. Stimulates melanin elimination through the keratinocytes: Placental protein, Azelaic acid
4. Direct reduce melanin (Cytotoxic effect on melanocytes): The most known active ingredient based on this mechanism is hydroquinone. Hydroquinone is still one of the most popular ingredients used in some countries. It inhibits the conversion of DOPA to melanin by inhibiting the tyrosinase enzyme. Other proposed mechanisms are inhibition of DNA and RNA synthesis, degradation of melanosomes and destruction of melanocytes (Zhai and Maibach, 2001). It has been reported to cause serious side effects if applied over longer periods of time. Moreover it leads to permanent de-pigmentation, and as a result of that, it increases photosensitivity of the skin. For that reason hydroquinone is banned for use in cosmetic products in most of the countries and/ or permitted only at limited concentrations in cosmetic formulations (Zuidhoff and Rijsbergen, 2001).
5. Tyrosinase inhibitors

5.1 Suppressive type: Only a few active ingredients are currently known to have tyrosinase formation suppression abilities. The best known active is kojic acid (gamma-pyron compound). Kojic acid, acting directly on the activity of the enzyme, includes a number of copper-chelation agents and suppressing the tautomerization from DOPAchrome to DHICA, it also act as reducing agents on melanin intermediates, thus blocking the oxidation chain reaction at various points from tyrosine and DOPA to

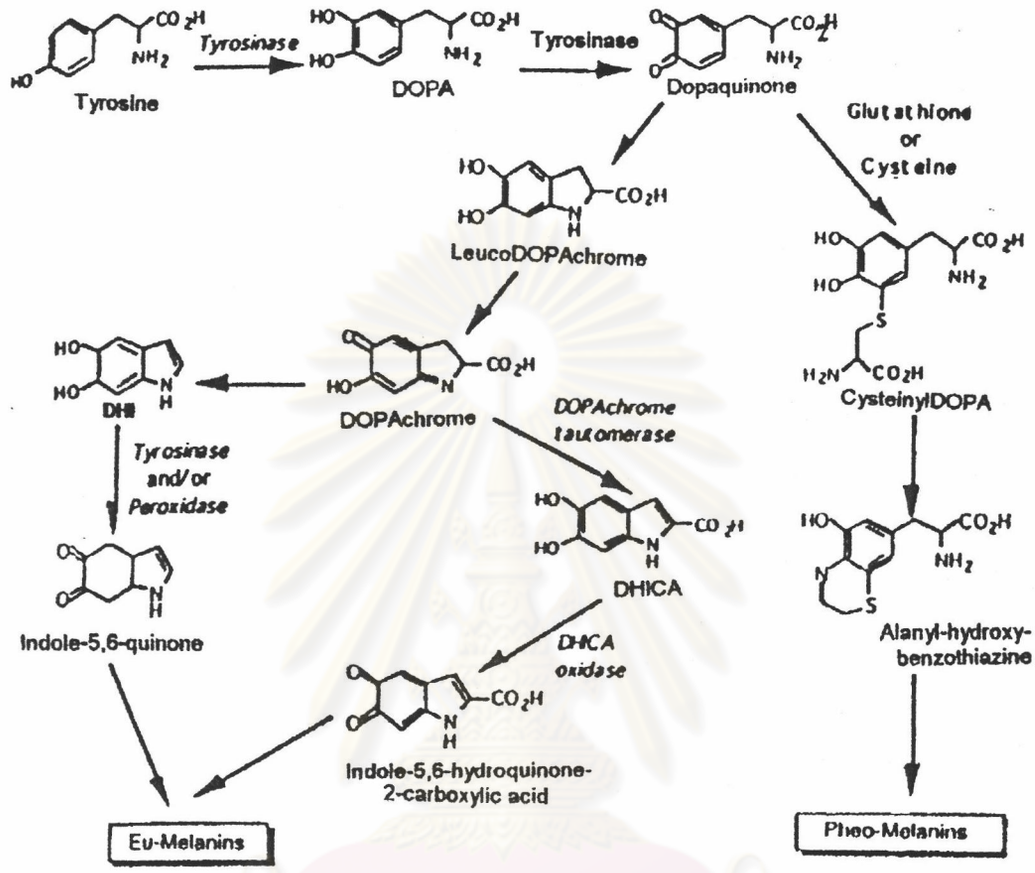

Figure 5 Melanin-biosynthesis pathways in melanocytes

melanin (Figure 5). Kojic acid proved to be the most useful one for cosmetic applications. Its depigmenting properties have been extensively studied by Kose© (Japan), where the product was first commercialized. According to Zhai and Maibach (2001), kojic acid, a fungal metabolic product, is increasingly being used as a skin whitening agent in skin care products marketed in Japan since 1988. It was first isolated from *Aspergillus* in 1907. Kojic acid is used in concentrations ranging from 1 – 4% (Tibibian, 2000).

5.2 Non-suppressive type: Skin whitening ingredients that can inhibit the activity of tyrosinase are in general highly soluble in water. Their mechanism is based on an alteration of the active center of the enzyme tyrosinase, thus reducing its activity. In most cases the ingredients are extracted from nature sources, such as bearberry (arbutin) leaves, licorice root (glycyrrhizinic acid) and citrus fruits (ascorbic acid). The tyrosinase inhibitors mentioned basically are all reductants and utilize their reducing power. The disadvantage of these ingredients is in general their high price and instability in formulations (in some cases).

Licorice extract

Glabridin (glycyrrhizinic acid) is the main ingredient in licorice extract. Licorice extract is extracted from the *Glycyrrhiza glaba.* Its has been used as whitening in cosmetic preparations (Yokota, Nishio, Kubota 1998). Tibabain, (2000) showed that UVB-induced pigmetation and erythema were inhibited by topical application of 0.5 % glabridin.

Puag-Haad

Puag-Haad is dried aqueous extract of the heartwood of *Artocarpus lakoocha* and its activities come from 2,4,3′,5′-tetrahydroxystilbene (Poopyrunchpong et al., 1978; Farnsworth and Bunyapraphatsara, 1992). Puag-Haad usually appeared in the market as a brown lump. It was prepared by boiling chips of heartwood of *Artocarpus lakoocha* in water and the aqueous extract was concentrated by gentle heat. On cooling a yellow-brown powder of the Puag-Haad was separated. The precipitate was filtered and dried near the fire (Mongolsuk, 1975). *A. lakoocha* is not common in evergreen forest in the Penisular, southeastern and southwestern parts of Thailand. They are cultivated for

medicinal uses. The claimed efficacies in Thai traditional textbooks are as follows (Farnsworth and Bunyapraphatsara).

Roots: as an antipyretic, anthelmintic; for alleviation of toxic symptoms and treatment of urinary stones

Wood: as antiflatulence carminative and laxative; treatment of skin rash, chronic gastrointestinal ailments of children between the ages of 5 and 13 characterized by marked malnutrition and usually associated with intestinal parasitism, round worm infestation, menstrual disorders, fainting, tapeworm, any disorders or diseases which cause cachexia, disorders of flatulence and tendomyopathy

Bark: as antipyretic

Pith: treatment of menstrual disorders, any disorders or disease which cause cachexia, nephropathy, distension of abdomen due to peritonitis or paralytic ileus, insomnia, malnutrition syndrome in children due to intestinal parasitism, splenomegaly, eye irritation, dissipate hematoma, oropharyngeal symptom from gastroenteric disease, dyspepsia caused by wind element, cramp, clouded mind, incontinent urination; as antidiarrheal, anthelmintic, taenifuge, antituberculosis, analgesic and for increasing appetite

Evaluation of skin whitening agents

1. *In vitro* evaluations

To develop effective ingredients, including skin whiteners, reliable evaluation methods are essential. Skin whiteners are no exception. Certain tests should be used to determine whitener effectiveness, such as the enzyme, cell-culture and tissue-culture methods.

1.1 Enzyme method (Tyrosinase inhibitory activity): Tyrosinase, commonly taken from mushrooms, is added to its substrate tyrosine or DOPA, with or without skin whiteners. The agent's inhibitory activity is then evaluated by measuring the melanin intermediates by absorbance. Radioactive substrates can also be used (Masamoto et al., 1980; Masuda et al., 1996).

1.2 Cell-culture method: skin whitening agents are added to a culture medium to evaluate their inhibitory effect on melanogenesis in either mouse B-16 melanoma cells or

human melanocytes. This method allows detection of not only any inhibitory effect on the enzyme activity of tyrosinase, but also on any processes not related to tyrosinase, such as the inhibition of synthesis of the sugar side chain or the transfer of enzymes between organelles (Maeda and Fukuda, 1991).

1.3 Tissue-culture method: This method is sometimes used to study the interaction of melanocytes with their neighboring cells and tissues. Dissected mouse follicles or guinea pig skin are commonly used (Masuda et al., 1996).

2. *In vivo* evaluations

Once results from *in vitro* methods prove promising, *in vivo* methods should be used to confirm the agents' efficacies.

Measuring skin color

According to Wiechers et al. (1998), visual assessment of skin color is a subjective process that is highly dependent on the skill and experience of the operator. The development of instrumental techniques designed to objective quantification of skin color. Measurements are commercially available to the cosmetic scientist such as the Chromameter, the Mexameter, the Erythemameter and the Dermaspectrometer.

The Chromameter series of colorimeters are general-purpose industrial instruments. Results are expressed according to the Commission Internationale de Eclairage (CIE) color space definition where the L value a measure of overall reflectance across the color spectrum and is therefore, commonly used to determine lightness or darkness of skin. The Mexameter, Dermaspectrometer and Erythemameter have been developed that are specifically designed to measure the melanin and erythema content of skin by comparison of reflected light of different wavelengths. The experience and preference of the individual scientists therefore, often dictate the selection of an instrument for a particular study.

2.1 Preliminary screening tests: The classical method of measuring effect of chemicals is to use black-skinned animals. Such as guinea pig, cat or mice which do not pose very great problems in rearing, handling and testing (Hemsworth, 1973). It is also generally agreed that the skin of the guinea pig has enough similarity to human skin to make comparisons meaningful. Many of skin whitening agent's early screening tests were in fact carried out on black guinea pig and many agents were examined for their

depigmenting effects on these animals. However, the interpretation of the results of these tests was difficult since the degree of lightening could not be extrapolated to humans. It was decided, therefore, to evaluate skin whitening agents first of all on black guinea pig, then on black pig, and finally on a panel of humans. This was considered necessary in order to ascertain the usefulness of guinea pigs as a screening technique by correlating the results with those obtained on humans (Curry, 1974).

2.2 Effect on UV induced pigmentation: Following UV irradiation, pigmentation changes on exposed areas of either the shaved backs of guinea pigs or the human forearm are assessed visually, optically (by Chromameter or Mexameter) and histologically. Topical application of the test samples may start before or after the UV induced pigmentation has subsided, depending on the test's objective.

2.3 Clinical tests (Clinical trial): Placebo-controlled clinical tests determine a skin lightener's efficacy to cure or improve skin pigmentary disorders. Monitoring changes in skin color after topical application of test materials to UV-irradiated human skin. In some tests, subjects have melasma, freckles or senile lentigo. Researchers can also evaluate possible side effects and any other effects, such as the result of adding sunscreens (Lee and Kim, 1995).

Antioxidants

The stability of cosmetic dosage forms may refer to the stability of physical and chemical forms (Mithal, 1980). Their commonest role is in the preservation of products that are particularly susceptible to oxidation, with the formation of objectionable degradation products of unpleasant odour. Fortunately this aspect can be taken care of by inclusion of some additives known as antioxidants. The antioxidants have appreciable affinity for oxygen and when they are included in a formulation they compete for it affording protection to other oxygen sensitive compounds (Carter, 1975). Antioxidants are categorized as 1) True antioxidants that function by breaking the free radical chain. They are effective against oxidation by atmospheric oxygen (autooxidation), for example, 0.01 - 0.1% butylated hydroxyanisole (BHA), 0.005 - 0.15% propyl gallate and 0.01 - 0.1% tocopherols (Boylan, Chowhan and Cooper, 1896; Smolinske, 1992). 2) Reducing agents are not preferentially oxidized but act by

blocking an oxidative chain reaction, for example, 0.01 – 0.15% sodium metabisulfite and 0.01 – 0.02% sodium sulfite (Carter, 1975). 3) Synergists, which generally have little effect themselves but enhance the action of true antioxidants either by removing pro-oxidant metals or by regenerating the antioxidant by reduction. For example ethylene diaminetetraacetic acid (EDTA) and calcium EDTA are chelating agents, they form stable water soluble complexes (chelates) with alkaline earth and heavy metal ions. The chelated form has few of the properties of the free ions, and for this reason chelating agents are often described as "removing" ions from solution (also called sequestering). The stability of the metal-EDTA complex depends on the metal ion involved and also on the pH. Antioxidant synergists have been used both alone and in combination with true antioxidants. Concentrations in the range 0.005 – 0.1% have been employed (Boylan et al., 1986).