CHAPTER II

LITERATURE REVIEWS

Artocarpus incisus (Breadfruit)

1. Botanical Properties

Scientific Name : *Artocarpus incisus*

Related Name : *Artocarpus altilis; Artocarpus communis* Forst.; *Artocarpus incisus* (Thunb.) L.

Common Name : Sa-ke, Khanun-sampalor (Thai)

Family : Moraceae

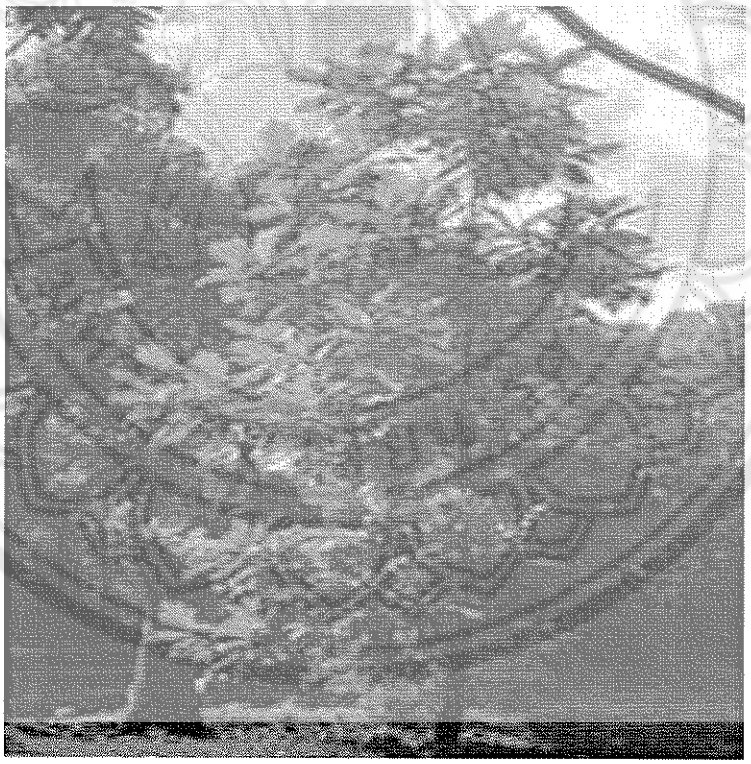


Figure 1 ***Artocarpus incisus***

Source: www.destinationtropicals.com

2.General characteristics

Artocarpus incisus (breadfruit) is called "Sa-ke" in Thai. It is found throughout the tropical area including Thailand. This evergreen tree is a large tree, reaching heights of 15-20 m; smooth bark and light colored. It will be productive for 50 years or more. The leaves are bright-green and glossy on the upper surface, with conspicuous yellow veins; dull, yellowish and coated with minute, stiff hairs on the underside. Breadfruit is disseminated with the root cuttings and yields its fruit up to 2-3 times in a year. The fruit produces 1-4 kg. White milky latex is present in all parts of the tree. The huge trunk may attain 2-3 meter circumference depending on the variety, it either slightly widens at the base or forms narrow buttresses [10]. A characteristic of breadfruit is shown in Figure 1.

3. Traditional uses

Breadfruit is rich in carbohydrates (76.7%) and nutritional energy [11]. Their trees are also a good source of medicine, insecticides, adhesives, timber and shelter [12]. In Trinidad and Bahamas, a leaf extract obtained from decoction is believed to lower blood pressure, and is also said to relieve asthma. Crushed leaves are used on the tongue as a treatment for thrush. Leaf juice is applied as ear-drops. Ashes of burned leaves are used in skin infections. Powder of roasted leaves is employed as a treatment for enlarged spleen. Crushed fruit is poultice on tumors to "ripen" them. Toasted flowers are rubbed on the gums around an aching tooth. Latex is used on skin diseases and is bandaged on the spine to relieve sciatica. Diluted latex is taken internally to overcome diarrhea [13].

The heartwood of *A. incisus* has been reported that their components exhibit strong inhibition on tyrosinase activity and melanin biosynthesis [3, 4]. The methanol extract of the heartwood of *A. incisus* grown in Okinawa, Japan, strongly inhibits tyrosinase activity without any cytotoxicity [4]. Additionally, artocarpin obtained from crystallization of *A. incisus* ether extract also shows melanin biosynthesis inhibitory effect on brown guinea pig without skin irritation [5].

4. Chemical compounds

The heartwood extract of *A. incisus* consists of several flavonoids including artocarpin, (+)-norartocarpin, artocarpesin, (+)-dihydromorin, chlorophorin, (+)-norartocapanone, artocarbene, 4-prenyloxyresveratrol and cycloartocarpin. In addition, they reported that chlorophorin, (+)-norartocapanone, artocarbene and 4-prenyloxyresveratrol show much higher tyrosinase inhibitory activity than kojic acid [4]. The chemical structures of the active compounds from *A. incisus* are shown in Figure 2. Furthermore, a major component of the extracts, artocarpin [6-(3-methyl-1-butenyl)-5,2',4'-trihydroxy-3-isoprenyl-7-methoxyflavone] shows distinct skin lightening effect on the UVB-induced hyperpigmented dorsal skin of brownish guinea pigs [5]. Recently, there has been reported that the potential of tyrosinase inhibition of *A. incisus* ether extract was closed to that of kojic acid based on the IC_{50} values from DOPAchrom assay (IC_{50} values of kojic acid and the extract were 10.26 and 7.89 μg/mL, respectively). Nevertheless, kojic acid showed lower inhibitory activity on melanogenesis than the ether extract in mouse B16F1 melanoma cell. Additionally, the dendrite morphology of melanocyte B16F1 treated with kojic acid and hydroquinone were changed whereas the ether extract did not affect on the melanocyte morphology [3]. These findings lead to interesting for development of the whitening product from the *A. incisus* heartwood extract.

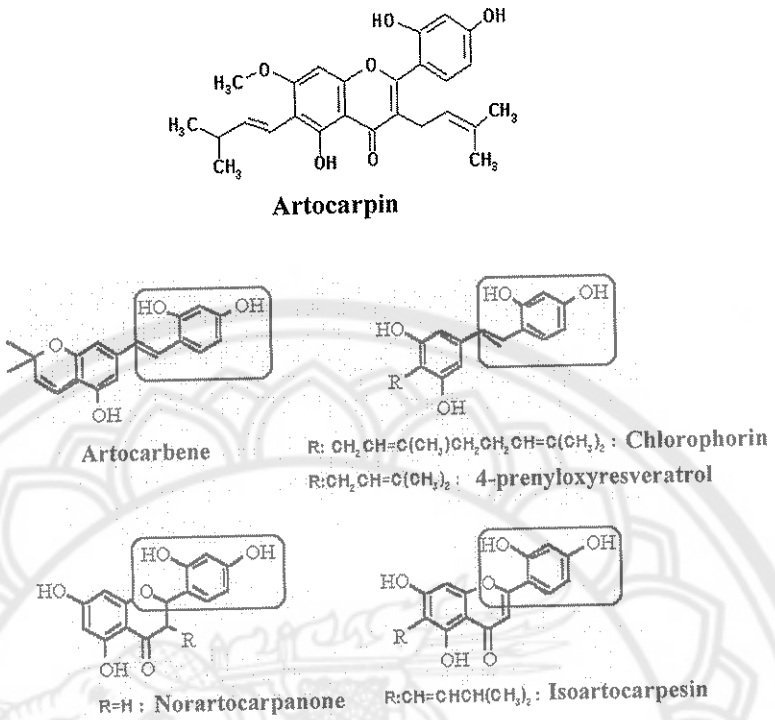


Figure 2 Chemical structure of active compounds isolated from *A. incisus* heartwood extract.

Skin structure

Skin is the largest organ of human body. It is an essential interface between a living organism and its environment, reacting to various stimuli in that environment. The integument not only physically protects the internal organs and limits the passage of substances into and out of the body but also stabilizes temperature and blood pressure through its circulation and evaporation systems. The skin consists of three layers: epidermis, dermis and hypodermis, as shown in Figure 3. Each layer has particular function, but works in coordination with the next layer [14].

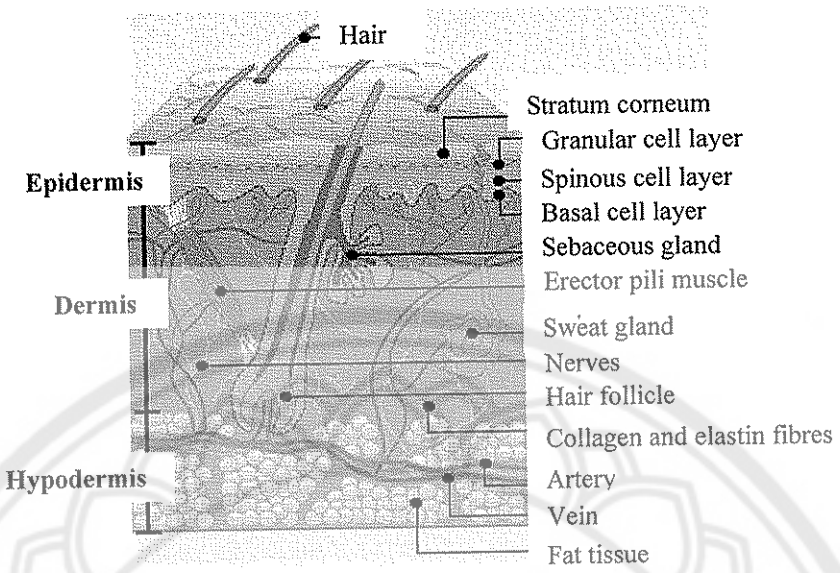


Figure 3 Skin structure

Source: www.razor-gator.com/.../skinstructure.htm

Epidermis

Epidermis is the outermost layer of the skin. Therefore, it is in close contact with the environment that serves as a protective barrier. It contains several small organs of varying density depending on the location: hair, sebaceous glands, eccrine sweat glands, appocrine glands and neurosensory organs. Thickness of the epidermis varies with age, sex and the location on the body. In most parts of the body the epidermis is about 0.1 mm thick but on the palm and sole it may be 0.8-1.4 mm thick. In addition, the majority of epidermal cells are keratinocytes (90-95%) that synthesize protein called keratin [15]. Keratin is a structural that is specific to the skin, hair and nail that of epidermis are death cells. Acidic and basic keratins make up about 80% of the dry mass of the corneocytes [16]. The epidermis consists of different five layers, as shown in Figure 4.

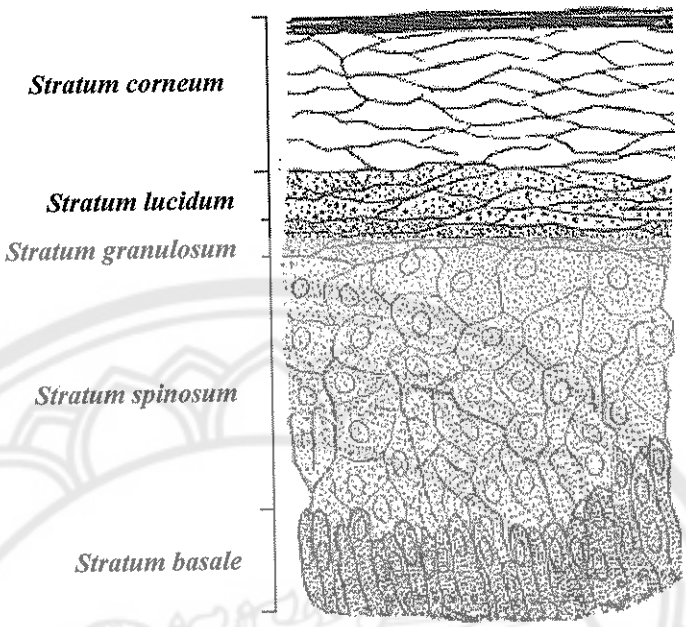


Figure 4 Epidermis structure

Source: http://commons.wikimedia.org/wiki/File:Skinlayers.png

1. *Stratum basale (Stratum germinativum)*

The basal cell layer is the lowermost layer of the epidermis adjoining the underlying dermis. It is also a single layer of cell each of which is a capable of cell-division [17]. This layer is comprised mostly of keratinocytes which are either dividing or non-dividing. The cells contain keratin tonofibrils and are secured by hemidesmosomes to the basement membrane [18].

Melanocytes make up to 5-10% of stratum basale and produce melanin which is transferred to neighboring keratinocytes via dendritic processes. The melanocytes are of neural crest origin and most numerous on the face and other exposed areas of the skin [19]. The merkel cells can also be found in this layer and are closely associated with terminal filaments of cutaneous nerves. Merkel cells have a role in sensation. Neuropeptide granules, neurofilaments and keratin can be seen in their cytoplasm.

2. *Stratum spinosum*

Daughter basal cells migrate upwards and differentiate into polyhedral cells in this layer. Desmosomes interconnect these polyhedral cells and give rise to the "prickles/spines" seen at light microscope level. Keratin tonofibrils form the cytoplasmic supportive network. Langerhans cells are mostly found in this layer. They are dendritic, immunologically active cells that play a role in antigen presentation [19].

3. *Stratum granulosum*

In this layer, cells become flattened and lose their nuclei. In the cytoplasm, there are keratohyalin granules as well as membrane-coating granules which expel their lipid contents into the intercellular spaces [20].

4. *Stratum lucidum*

The stratum lucidum is normally only well seen in thick epidermis, in the palm of hand and the sole of the foot, and represents a transition from the stratum granulosum to the stratum corneum. It is composed of three to five layers of dead, flattened keratinocytes [21].

5. *Stratum corneum*

The stratum corneum is composed of sheets of overlapping polyhedral cornified cells with no nuclei called corneocytes which are terminally differentiated, microscopically flat and tightly packed in stacks that run perpendicular to the skin surface [22]. This layer is non-viable layer that is approximately 10-20 μm thick [23]. It is thickest on the palm and sole. The flattened corneocyte develops a thickened cell envelope.

Its cytoplasm is replaced by keratin tonofibrils in a matrix formed from keratohyalin granules. Whereas, the membrane-coating granules produce lipid glue that keeps the cells stuck together. This forms the main barrier in which a number of processes need to be taken into account including partition and diffusion within and through layer, metabolic processing and the systemic circulation [16].

Dermis

Dermis is connected to the epidermis. It is a well-vascularized connective tissue also permeated by nerve fibers and lymphatic vessels. A structure of this layer is fibrous network of proteins. These proteins are made up for the most part of collagen fibers (75%) while elastin and reticulin fibers appear only in limited amounts [24]. Due to its high water retention properties, these fibrous proteins are imbedded in a colloid base, thus, it is responsible for the turgidity of the skin. The main cells present are the fibroblasts and melanocytes. The dermis dose not only provides the nutritive, immune and support systems for epidermis, but also plays a role in temperature, pressure and pain regulation [16].

Subcutaneous

Subcutaneous (hypodermis) is the deepest layer of the skin. This layer consists of adipose or fatty tissue arranging in lobules and linking to the dermis by interconnecting collagen and elastin fibers. The fatty tissue of this layer provides nourishment to the dermis and upper layers of skin. Furthermore, it also conserves body heat and cushions internal organs against trauma [23]. Blood vessels, nerves, sweat glands and deeper hair follicles are found in this layer [25].

Skin color

Skin color or skin pigmentation is one of the most visible indicators that help distinguish human appearance. It is regulated by complicated processes which results from the synthesis and distribution of melanin. The skin color involves the co-operation of melanocytes and keratinocytes in producing melanosomes and then transfers them to keratinocytes, which then distribute them in various fashion routes to the surface of the skin [26]. Additionally, the most important of the skin color is the activity of melanocyte: the quantity and quality of pigment production, not the density of melanocytes [27]. In fact, the number of the melanocyte is similar in all nationality. Nevertheless, the melanocytes of darkly pigmented skin have thicker, longer and branched dendrites. The difference in skin and hair color is determined by a number of factors of which the most important are the degree and distribution of melanin pigmentation [28].

1. Melanocyte function

Melanocytes are located in the basal layer, as shown in Figure 5. In this location, they produce melanin pigment in elongated, membrane-bound organelles known as melonosomes. Melanin is packaged into granules which are moved down by dendritic processes and transferred by phagocytosis to adjacent keratinocytes. The association of melanocyte and its surrounding keratinocytes has often been defined as "epidermal melanin unit" where one melanocyte supplies about 36 keratinocytes with melanin granules [14]. The distribution of melanin in the epidermis is shown in Figure 6.

In the inner layers of the epidermis, melanin granules form a protective cap over the outer part of keratinocyte nuclei. In the stratum corneum, melanin granules are uniformly distributed to form a UV-absorbing blanket which reduces the amount of radiation penetrating the skin which results in a profound alteration of the metabolism, structure and function of melanocyte [29].

Contrary to popular belief, variations in racial pigmentation are not due to differences in melanocyte numbers, but to the number and size of melanosomes produced.

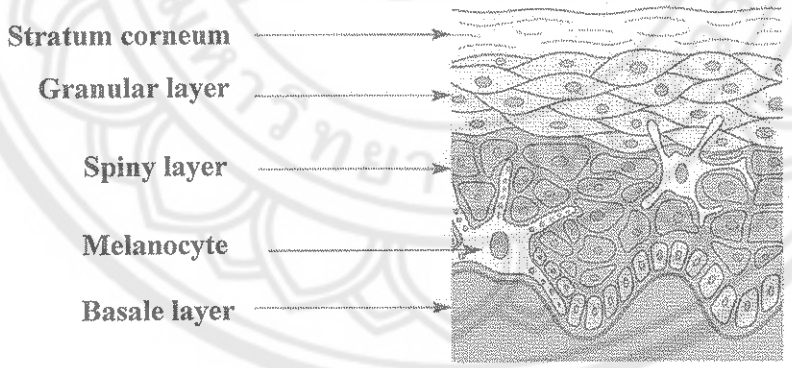


Figure 5 The melanocyte

Source: www.pg.com/science/skincare/Skin_tws_16.htm

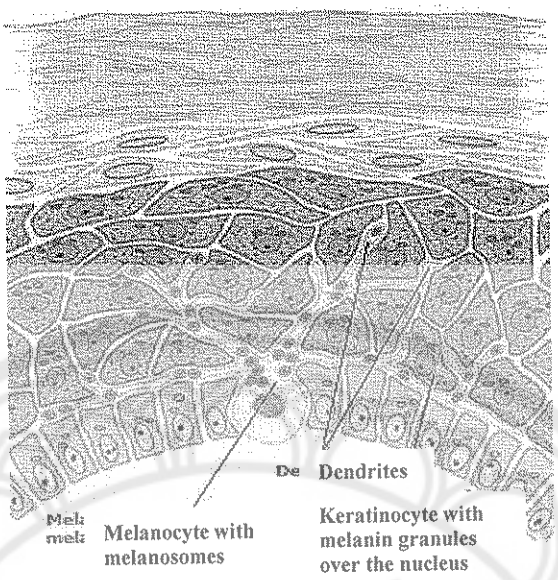


Figure 6 The distribution of melanin in the epidermis

Source: www.pg.com/science/skincare/Skin_tws_16.htm).

2. Melanin biosynthesis

Visible pigmentation in human such as in the skin, hair bulbs and eyes results from the synthesis and distribution of melanin. Recent studies have shown that melanin not only functions as sunscreen to absorb UV and prevent DNA damage but also play important roles in the other properties such as antioxidant and radical scavenger [26]. Melanin synthesis is regulated by melanogenic enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2). Originally, the production of the melanin biopolymer was thought to only one copper-containing enzyme known as tyrosinase [27]. The melanin pigments, both the brown-black eumelanin and the yellow-red pheomelanin, are all derived by oxidation of the amino acid tyrosine [28]. In addition, the level and type of melanin production relate to the activity of the various enzymes as well as MSH (α-melanocyte stimulating hormone), agouti signaling protein, basic fibroblast growth factor (bFGF), endothelin-1 and ultraviolet light [23]. The melanin biosynthesis pathway is shown in Figure 7.

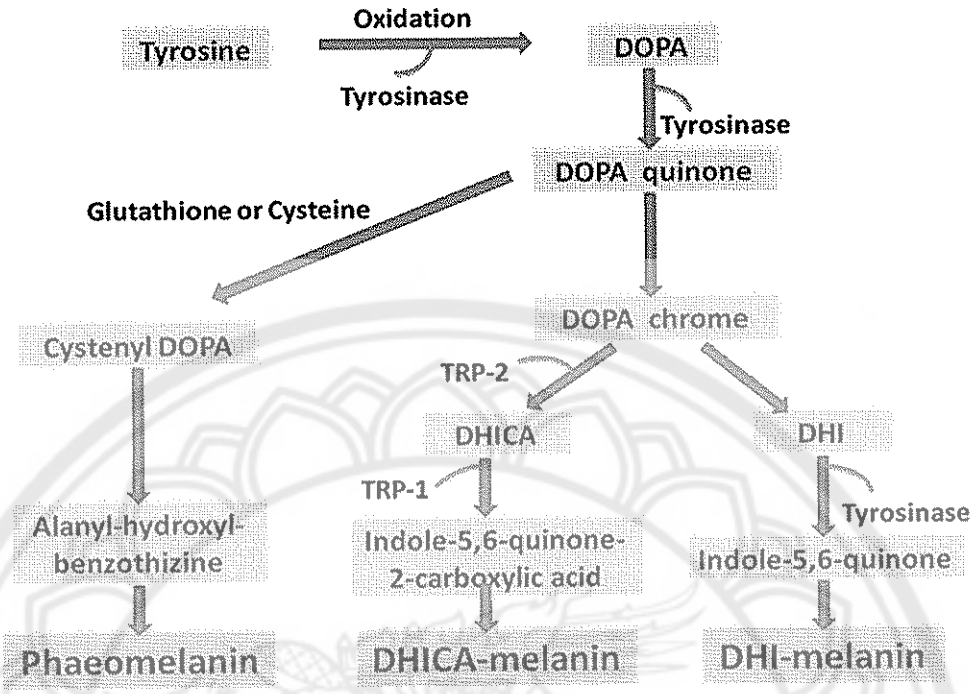


Figure 7 The melanin synthesis pathway [24].

The initial step in the melanin synthesis is controlled by enzyme tyrosinase which oxidizes amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA). DOPA spontaneously autooxidize to DOPAquinone without tyrosinase. DOPAquinone is an extremely reactive compound that is the absence of thiols in the reaction medium, undergoes intramolecular cyclization leading to leukodopachrome and then to DOPAchrome. DOPAchrome decarboxylates spontaneously to 5,6 dihydroxyindole (DHI). In the presence of divalent cations and the enzyme DOPAchrome tautomerase, also called tyrosinase related protein 2 (TRP-2), the intermediate 5,6-dihydroxyindole-2-carboxylic acid (DHICA) will result. DHI is oxidized to indole-5,6-quinone while DHICA is oxidized to indole-5,6-quinone-2-carboxylic acid. It is speculated that the oxidation of DHICA is catalyzed by an enzyme called DHICA oxidase which is synonymous with tyrosinase related protein 1 (TRP-1) and the oxidation of DHI by tyrosinase. The quinones are thought to build melanin by oxidative polymerization. Whether this polymerization step is under enzymatic control is not yet clear.

Melanins generated from DHICA are brown polymer, poorly soluble and of intermediate weight, whereas those generated from DHI are black, totally insoluble and of high molecular weight. These melanins are termed eumelanins. Eumelanins are a mixture of DHI- and DHICA-melanins and the chemistry of these pigments may vary to a considerable extent [29]. In the presence of sulfhydryl donors, probably cysteine, DOPAquinone is converted to cystenyl DOPA. Further oxidation, cyclization and polymerization leads to the formation of pheomelanin. Pheomelanins have a yellowish-red color, are soluble in alkali and have a low molecular weight. It is synthesized in smaller amount compared to eumelanin [30]. These different types of melanin are responsible for the differences in hair color in mammals and in man.

UV induced pigmentation

Of the total solar energy reaching the surface of the earth, about 56% is in the infrared range and 39% is the visible light. However, it is the remaining 5%, the ultraviolet (UV) light which has the most profound effect on the human skin. The UV radiation is divided as follow:

1. UV-C radiation (100-280 nm): This radiation is the most dangerous radiation. It is a sterilizing ray that is carcinogenic and can kill small organisms on contact. Fortunately, it is virtually completely screen out by the Earth's atmosphere, and is thus a negligible source of adverse human health effects [29].

2. UV-B radiation (280-315 nm): UV-B ray is the most active UV radiation for producing sunburn, and penetrates into the epidermis of the skin. It is considered to be responsible for inducing skin cancer due to DNA damage [30]. It is also suspected of lowering the skin's immune defense system. However, it is crucial in the synthesis of vitamin D, which some recent studies suggest may potentially reduce risk of colon, prostate, and breast cancers [29].

3. UV-A radiation (315-400 nm): It is the longest wavelength and makeup 95% of UV light. The longer wavelength penetrates deep into the epidermis and dermis of the skin. UV-A is responsible for skin aging, wrinkling and loss of elasticity. In addition, it is involved in the generation of singlet oxygen and hydroxyl free radicals which can cause damage to cellular proteins, lipid and carbohydrates [31]. It also increases the damaging effects of UV-B, including skin cancer and cataracts.

After sun exposure, the melanin pigment is produced in the melanocytes of epidermis and then migrates to the epidermal cells within 24-48 hours. The differences in radical skin pigmentation depend on the quantity of pigments produced and the deposition of these pigments throughout the epidermis. One of the most important protection mechanisms of the human skin against radiation is the production of melanin which is crucial in the absorption of free radicals generated within the cytoplasm and in shielding the DNA in the nucleus of the epidermal cells from UV and visible radiation damage [27]. However, the over strain of the protection of the body's mechanisms lead to erythema. The erythema is a critical reaction to radiation, the border at which a burn develops. Since the action spectrum of sunburn and melanogenesis are virtually identical, it is assumed that they are induced by the same mechanism [32].

Depigmentation and skin whitening agents

1. Depigmentation

The most common pigment abnormalities in the skin of color are post-inflammatory hyperpigmentation, melasma and sun-induced hyperpigmentation [33]. Depigmentation can be achieved not only by biological and chemical, but also by physical treatments [34]. Since the introduction of hydroquinone as a skin lightening agent in 1961, several products with depigmenting properties have been used for the treatment of pigmentary disorders of the skin [35].

The ideal depigmenting agents should have a potent depigmenting effect with a rapid time of onset (less than 2 to 3 months), carry o short- or long- term side effect and lead to a permanent removal of undesired pigment [36]. Depigmentation can be achieved by regulating (i) tyrosinase inhibition, maturation and enhancement of its degradation; (ii) transcription and activity of tyrosinase, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2) and peroxidase; (iii) interference with melanosome maturation and transfer; (iv) melanocyte loss [37]. However, as a result of the key role is played by tyrosinase in the melanin biosynthesis, most whitening agents act specially to reduce the function of enzyme by mean of several mechanisms; (i) interference with its transcription and/or glycosylation, (ii) inhibition by different modalities , (iii) reduction of by-products, and (iv) control of post-transcriptional [6].

2. Skin whitening agents

Hydroquinone (HQ) is a well-known tyrosinase inhibitor. Besides tyrosinase inhibition through interaction with copper at the active site, alteration of melanosome functions, depletion of glutathione, generation of reactive oxygen species and subsequent oxidative damage of membrane lipids and proteins may play role in the depigmenting effect of HQ [6]. It has been frequently used for many years, mixed with other compounds to increase its efficiency [38] in a number of melasma treatments, but today the human use of HQ has important legal restrictons. However, HQ is considered to be highly cytotoxic to melanocytes and potentiality mutagenic to mammalian cells [39]. These adverse effects of HQ have led to the search for safer and natural-based skin whitening products. Therefore, the ideal agent for whitening products is the one that inhibits melanogenesis without cytotoxicity. The currently available whitening agents are shown in Figure 8.

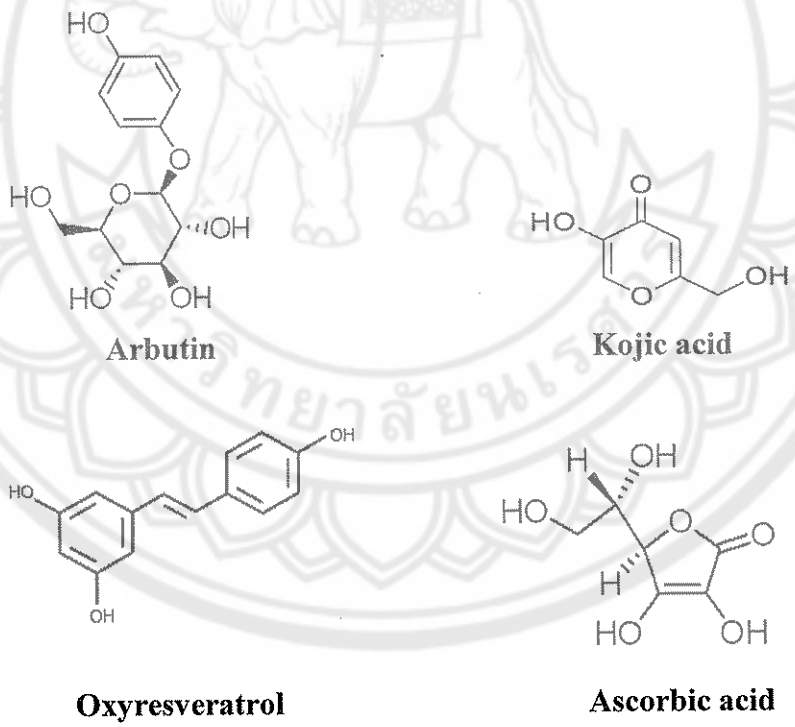


Figure 8 Chemical structures of whitening agents.

Arbutin, a natural β-glucoside of hydroquinone, is commonly used. It inhibits not only tyrosinase but also melanosome maturation, possibly by its reported actions on DHICA polymerase activity and the silver protein in this organelle [40]. As one of the arbutin derivatives, α-arbutin has been developed and commercialized in the cosmetic industry [41].

Kojic acid is a natural substance produced by fungi and bacteria. It is a potent tyrosinase inhibitor and it inactivates tyrosinase by chelating with its vital copper ion. Kojic acid also suppresses the tautomerization from DOPAchrome to DHICA (TRP-2). However, it may cause allergy [42].

Oxyresveratrol is the compound of mulberry (*Morus alba* L.) extract. It inhibits tyrosinase activity remarkably, shows no toxicity and successfully passes a number of tests including the most sensitive ones such as eye and human skin irritation test [44].

L-ascobic acid (Vitamin C) and its derivatives are believed to act as reducing agents on melanin intermediates, thus blocking the oxidative chain reaction at various points from tyrosine/DOPA to melanin. In addition, it has a reducing effect on o-quinones and oxidized melanin. It can also alter melanin from jet black to light tan [36].

3. Melanin content assay

Melanin content assay is the determination of the melanin amount in melanocyte cells which are treated with the whitening agents. In the melanogenesis studies employ B16 melanoma cells which are useful in demonstrating several new mechanisms of melanogenesis inhibition. Potency of tested whitening agent is indicated by the cell concentration, cell morphology and the extracts of melanin pigment in cultured cell [45]. Whole melanin is determined by using two condition assays. The secretion of melanin in the medium is estimated from extracellular melanin content. On the other hand the intracellular melanin is quantified in the pelleted cells [46].

Antioxidant properties as potent tyrosinase inhibitors

1. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) include superoxide anions ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\bullet OH$), and nitric oxide. An accumulation of these ROS can result in oxidative stress that has been related to human diseases such as cardiovascular diseases, cancers, aging, diabetes and atherosclerosis including skin problems [47]. These molecules are extremely chemically reactive and short-lived. They react at the place where they are created. They are also dangerous when attacking biological molecules and leading to cell of tissue injury associated with degenerative disease [48]. Moreover, ROS enhance melanin biosynthesis, damaging DNA, and may induce proliferation of melanocytes [49].

2. Antioxidant and skin whitening effect

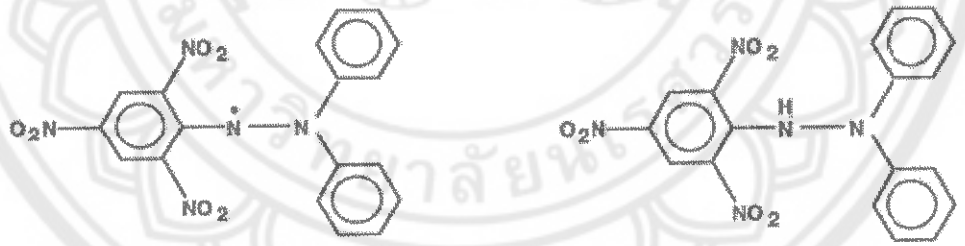
Antioxidants are introduced as primary ingredients in cosmetics to scavenge free radical produced by UV light and environmental pollutants [50]. It is known that ROS scavenger or inhibitors such as antioxidant may reduce hyperpigmentation [51].

Antioxidants such as phenolic compounds can scavenge free radicals, which induced by ultraviolet radiation, and may be effective as depigmenting agents [52]. On the basis of Huckel theory calculation for tyrosinase active site model, it has been shown that ionization of hydroxyl group of phenolic compound is a crucial step in its interaction with positively charged copper of the active site in monophenolase reaction [53]. Among the various phenolic compounds, the flavonoids are a class of plant phenolics with significant antioxidant and chelating properties. It is widely believed that the antioxidant ability of flavonoids resides mainly in their ability to donate hydrogen atoms and thereby scavenge the free radicals [54]. Many tyrosinase inhibitors are phenolic derivatives of flavonoids such as artocarbene, chlorophorin, and norartocarpanone. These inhibitors are usually constructed from 4-substituted resorcinol moiety and catechol structure which inhibit and may behave as a chelator to the copper ions in the tyrosinase. Additionally, these compounds can prevent pigmentation resulting from auto-oxidation processes [55].

3. DPPH assay

The stable radical species diphenyl-picrylhydrazyl (DPPH) has been widely used for antioxidant capacity screening. The DPPH assay is originally developed by Blois, which easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule as shown in Figure 9.

DPPH has been widely used for estimation due to its clear reaction mechanism, solvent compatibility and technical simplicity of its assay which requires no special equipment [56]. In addition, it has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition, brought about by various additives [48]. The purple colored DPPH has strong characteristic absorption at 515 nm and can undergo reactions with hydrogen donating antioxidant compounds to yield the stable yellow DPPH-H molecule easily monitored with UV spectroscopy [57, 58]. The degree of decoloration of this solution indicates the scavenging efficiency of the sample compounds. This assay can accommodate a large number of samples within a short period, and is sensitive enough to detect low concentrations of the principles [59, 60].



(A) Diphenyl-picrylhydrazyl (free radical) (B) Diphenyl-picrylhydrazine (nonradical)

Figure 9 Free radical form of DPPH (A) and reduced form of DPPH (B) after accepted an electron or hydrogen atom.

Post-inflammatory hyperpigmentation of skin

Normally, melanocytes work in close harmony with their neighboring cells in the epidermis. They are influenced by a variety of biological factors and environmental factors which increase the melanocytes activity. There has been reported that one of the biggest causative agents of hyperpigmentation is probably oxidative stress caused by UV light [61]. DNA fragments produced after UV exposure also stimulate melanogenesis. It is estimated that one hour of sun exposure at noon in a locality like Hong Kong may result in as much as 1,000,000 DNA damages. Cytokines, alpha-melanocyte stimulating hormone (α-MSH), and vitamin D are reported to be factors important in UV-melanogenesis [7]. Furthermore, solar radiation induces both acute and chronic responses in the skin. Sun burn, sun tan, generation of reactive oxygen species (ROS) and immonosuppression are acute responses. Wrinkles, lentigines, post-inflammatory hyperpigmentation are chronic responses.

The post-inflammatory hyperpigmentation of skin (PIH) presents as irregular, darkly pigmented spot arising in area of previous inflammation. Generally, it is most appear in darker skinned people. The several causes of the PIH are as the following:

1. The stimulation of melanocytes during inflammatory periods, usually called epidermal hyperdermelanosis

2. A variety of things: infection, allergic reaction, acne scaring, mediation reactions, trauma like burn or cuts, exposure to ultra violet light and phototoxicity

3. Affects the basal cell layer of the epidermis, making it as difficult to remove as a tattoo

In principle, the inflammation is a process involving multiple factors acting in a complex network. The ingress of leukocytes into the site of inflammation is crucial for the pathogenesis of inflammatory conditions [62, 63]. Neutrophils and macrophages are known to recruit and play pivotal roles in acute and chronic inflammation, respectively [64]. At the inflamed site, the recruited cells are activated to release many inflammatory mediators which elicit the initiation and maintenance of an inflammatory response, causing a change from the acute phase to the chronic phase of inflammation. Therefore, inhibition of the cellular reactions is one of the targets that are generally used as an *in vitro* model for anti-inflammatory testing. Thereby, the inhibition of pro-inflammatory cytokine release from macrophages has been used as

markers for *in vitro* tests for chronic inflammation because of the cytokines are a factor important in UV-melanogenesis.

Nanoemulsions

Nanoemulsions can be defined as oil-in-water emulsions, having a droplet size range between 50-1000 nm. Usually, the average droplet size is between 100 and 300 nm. Nanoemulsions have been characterized as being transparent or slightly translucent, depending on the particle size and on the difference in refraction index between oil phase and aqueous phase. Unlike microemulsion, nanoemulsions are only kinetically stable. However, the long-term physical stability of nanoemulsions is excellent, compared to macroemulsions. Nanoemulsions offer stability advantages over macroemulsions. They have dramatically smaller particle sizes and therefore offer higher stability against creaming or sedimentation because their diffusion rate is faster than their sedimentation rate. Additionally, nanoemulsions are used in various applications such as drug polymer, pharmarceuticals, agrochemical cosmetics and microelectronics [65]. Using nanoemulsion in industrial applications is very attractive due to several reasons, including, inter alia, the following: 1) The very small droplet size prevents creaming or sedimentation. 2) The small droplet size and hence the large surface area makes these systems suitable for efficient delivery of active components. 3) Nanoemulsions do not require high concentration of surfactants. These systems can be prepared using moderate surfactant concentrations (in the range of 4-8% wt.), as opposed to microemulsion [67]. Usually, the nanoemulsions is produced by the dispersion or high-energy emulsification methods which involve high shear mixing, high-pressure homogenization or ultrasonification [65]. Nevertheless, more recently a neat low-energy emulsification method has been developed by taking advantage of phase behavior and properties for promoting the formation of untra-small droplets. These low energy techniques include self-emulsification, phase transition and phase inversion temperature methods (PIT) [68].

Phase inversion temperature method (PIT)

The phase inversion temperature (PIT) method was introduced by Shinoda in 1964. It is a low energy emulsification technique which enables the formation of nanoemusions without the use of high shear forces. The PIT technique makes use of temperature sensitivity of ethoxylate group in non-ionic surfactants. These surfactants are hydrophilic at low temperatures and become more hydrophobic with increasing due to dehydration of the polyoxyethylene chains [65]. Thus, emulsions that use them change achieve a reversible phase inversion from o/w to w/o at a well-defined temperature, called the Phase Inversion Temperature (PIT). There has been reported that the mixture of glyceryl monostearate and cetostearylpoly(oxyethylene(12)glycol), the emulsifier is arranged in lamellar layers around the oil droplets that can stabilize emulsion droplets against coalescence, as was also proven for other emulsion systems [9]. Hence, cosmetics o/w emulsions with improved stability and small droplet size can be manufactured by producing a w/o emulsion at high temperature and cooling through a phase inversion.

The PIT process, on heating, a coarse o/w emulsion passes a microemulsion phase in a certain temperature range and inverts to a w/o emulsion. It is decisive that during phase inversion a single-phase microemulsion or lamellar phase forms in which the hydrophilic-lipophilic properties of emulsifier mixture are balanced, in order to keep the interfacial tension between the oil and water phase minimal [69]. It was found that this transition is reversible if the nanoemulsion is rapidly cooled resulting in finely dispersed o/w emulsion [67]. Therefore, a finely dispersed o/w emulsion is obtained by a heating-cooling cycle, in which a microemulsion phase is passed as shown in Figure 10. There has been reported that the advantage of the two-step PIT process is a time- and energy-saving [9]. Furthermore, the PIT process is simple and also inexpensive expenses because it is not achieved by applying high shear forces such as high-pressure homogenizer.

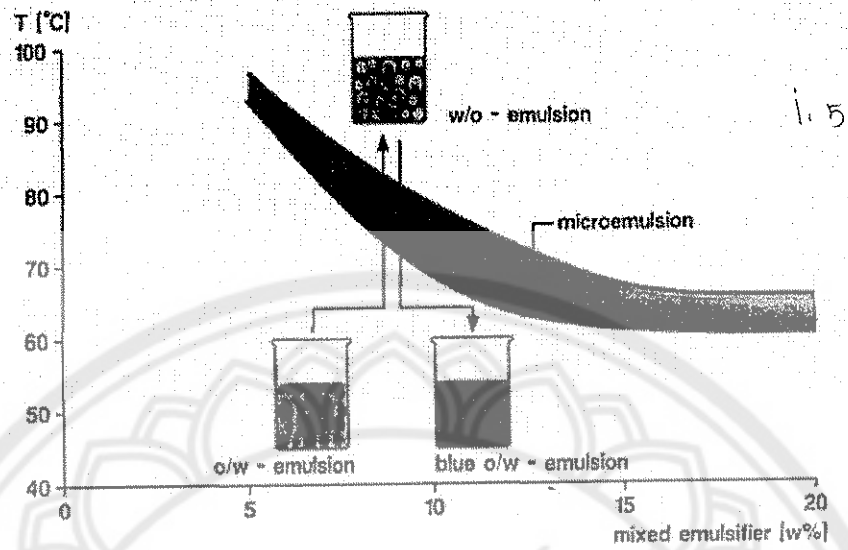


Figure 10 The principle of the PIT method.

Permeability pathway

There are two main pathways by which drugs can cross the skin and reach the systemic circulation (Figure 11). The more direct route is known as the transcellular pathway. By this route, drugs cross the skin by directly passing through both the phospholipid membranes and the cytoplasm of the dead keratinocytes that constitute the stratum corneum. Although this is the path of shortest distance, the drugs encounter significant resistance to permeation. This is because the drugs must cross the lipophilic membrane of each cell, then the hydrophilic cellular contents containing keratin, and then the phospholipid bilayer of the cell one more time. This series of steps is repeated numerous times to transverse the full thickness of the stratum corneum. Few drugs have the properties to cross via this method [70].

The more common pathway through the skin is via the intercellular route. Drugs crossing the skin by this route must pass through the small spaces between the cells of the skin, marking the route more tortuous. Although the thickness of the stratum corneum is only about 20 μm, but the actual diffusional path of most molecules crossing the skin is on the order of 400 μm[5]. The 20-fold increase in the actual path of permeating molecules greatly reduces the rate of drug penetration [70].

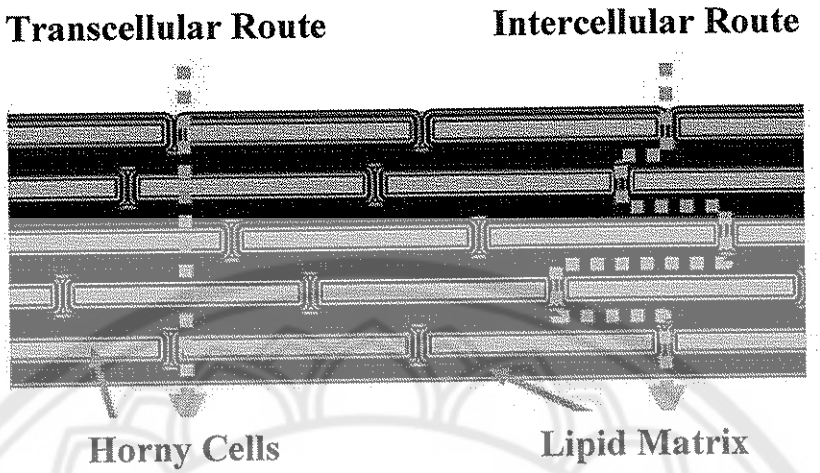


Figure 11 Transcellular and intercellular route

Source: www.scf-online.com

A less important pathway of drug penetration is the follicular route (Figure 12). Hair follicles penetrate through the stratum corneum, allowing more direct access to the dermal microcirculation. However, hair follicles occupy only 1/1,000 of the entire skin surface area. Consequently, very little drug actually crosses the skin via the follicular route as shown in Figure 12.

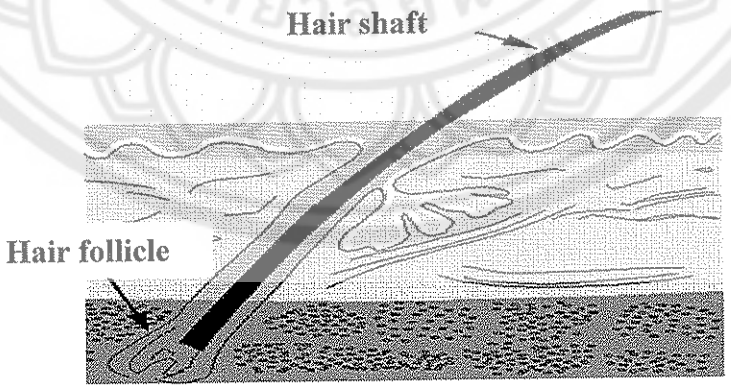


Figure 12 Follicular route

Source: www.bloombrook.com

***In vitro* evaluation of transdermal delivery**

The purpose of *in vitro* experiment in transdermal delivery is to understand and/or predict the delivery and penetration of active agent from the skin surface into the deeper skin of the living animal. Ideally, an *in vitro* system for transdermal delivery studies should be design, can be accurately determined. The release and skin permeation kinetics of active agent from transdermal delivery system can be evaluated by using a two compartment diffusion cell assembly under identical condition. This is carried out by individually mounting a skin specimen on a vertical or horizontal diffusion cell [71]. Each unit of transdermal drug delivery system is then applied with its active agent-releasing surface in intimate contact with the skin specimen. The skin permeation profile of active agent is followed by sampling the receptor solution at predetermined time and assaying active agent concentration in the samples by a sensitive analytical method such as high performance liquid chromatography [72]. The release profiles of active agent from these transdermal delivery systems can also be investigated in the same diffusion cell assembly without a skin specimen. Then the importance elements for *in vitro* evaluation of transdermal delivery are diffusion cell apparatus and skin model.

1. Diffusion cell apparatus

As regards transdermal delivery, it is well known that the main resistance to active agent transport resides in the skin, that is, diffusion through the stratum corneum. If an in vitro apparatus has poor mixing condition, the release rate from transdermal delivery system, which is usually much greater than the skin permeation rate, may be strongly distorted by the diffusion boundary layer. In the event, the in vitro release rate may become relatively close to the in vivo permeation rate, and it will be believed erroneously that the rate of active agent is controlled by the transdermal delivery system, not by the skin permeation. On the other hand, a well-designed in vitro apparatus can assure that the mechanism of active agent delivery is truly from the transdermal delivery system. Various types of in vitro apparatus for measuring active agent permeation profiles across the skin can be broadly divided into two categories as follow;

Physical design of diffusion cell	Method of sampling and measurement
Horizontal type	Continuing system
Vertical type	Fluid circulation system
Flow-through type	Noncirculation system
	Intermittent system : ratating agitation system

Vilia and Chien developed the horizontal-type skin permeation system. This cell design has a solution compartment of relatively small volume in each half-cell for maximal analytical sensitivity, and a rather small membrane area to accommodate the skin specimen available. Both the donor and receptor solutions are agitated, under a totally enclosed system. The temperature of the can be controlled isothermal or nonisothermal condition by circulating thermostats water through the water jacket surrounding the solution compartment.

The vertical-type skin permeation system is developed by Franz, Keshary, and Chien which has been frequently used for studying the kinetics of percutaneous absorption. The cell has a compartment with an effective volume and effective surface area. The solution in the receptor compartment is stirred by a rod shape magnet. The temperature in the bulk of solution can be maintained a constant level by circulating thermostated water through the water jacket surrounding the receptor compartment.

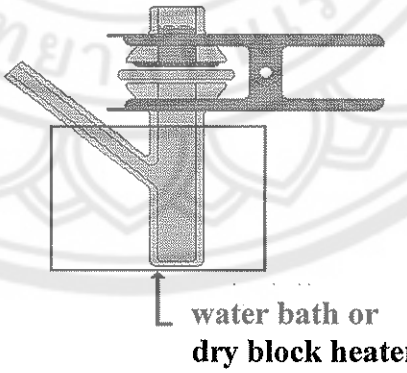


Figure 13 Franz-type diffusion cell apparatus

Source: www.permegear.com

Effect of diffusion cell apparatus on skin permeation was investigated by Keshary and Chien (1984). In the study, the release and skin permeation rates from four nitroglycerin transdermal therapeutic systems were evaluated in Franz and Keshary-Chein diffusion cell. The obtained result indicated that using the Keshary-Chein cell increase in the release and skin permeation rates comparative with Franz cell. These increases from the systems releasing nitroglycerin could be attributed to the improvements in the hydrodynamic conditions in the Keshary-Chien cell. These improvements result in a thinner hydrodynamic boundary layer, more efficient solution mixing, and better temperature control in the cell.

Thereby, in selection of diffusion cell apparatus, not only available dissolution equipment in laboratory is applied to reduce cost of experiment, but solution hydrodynamic, mixing efficiency, and temperature control are also considered to highest efficiency in *in vitro* permeation study.

2. Skin model

The great majority of *in vitro* experiments are conducted on animal skin. The use of human skin *in vitro* penetration studies is limited as human skin is often difficult to obtain, expensive, difficult to store, and full of variable in permeation properties. A variety of model membranes has been used for transdermal research such as hairless mouse skin, rabbit, rat and guinea pigs [73].The time of experimental of some animal skin in *in vitro* penetration studies is limited because of deterioration of membrane integrity after a prolonged use. In addition, most animal skin is more permeable than human skin partly due to a large number of hair follicles. Excised animal skin also has variable properties depending on preparation method and animal species. However, as comparing to an animal skin, the artificial membranes are limited because they lack of keratinized properties and lipid which are primary component in the stratum corneum of mammalian skins [74].

Evaluation of efficacy whitening products by scientific equipment

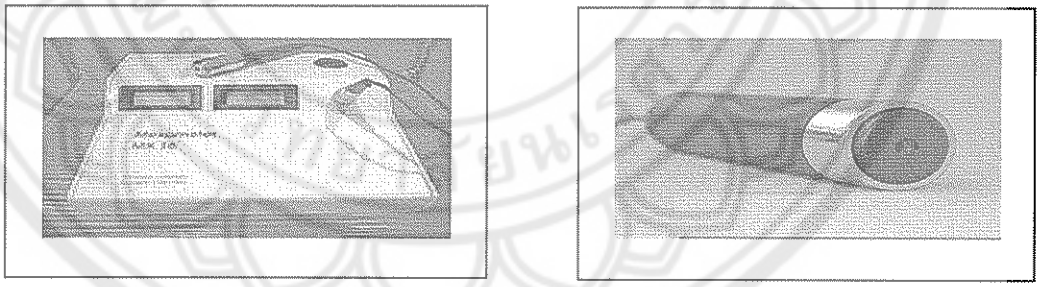
The effectiveness of whitening products is judged by the changing in the color of the pigmentation, the clearness of the boundary between the pigmentation and the surrounding skin, and the degree of the disappearance of pigmentation in the surrounding area of the skin. The skin reflectance (lightness) has been commonly used

as a measure of the evaluation of whitening or lightening effects. Nowadays, cosmeceutic skin care products encompass formulations of vitamin C, kojic acid, arbutin and ricorice. These substances have been claimed to exhibit whitening or lightening effect. The instruments used in this study are described as below.

Mexameter is a standard spectrophotometer designed to measure melanin and hemoglobin content (erythema) in the skin as an analysis of whitening response and determine skin phototype which is a classification system based on a person's sensitivity to sunlight. In addition, erythematic is used as an index of skin irritation and allergic reactions [66].

As show in Figure 14 (a), mexameter's probe emits light over a 5 mm diameter. It emits light of three wavelengths with 568,660 and 880 nm; green (568±3 nm), red (660±3 nm) and infrared (880±10 nm) [75].

The melanin value is measured with two wavelengths (660 and 880 nm) to achieve different absorption rates by melanin granules. For the hemoglobin measurement as well, two wavelengths (568 and 660 nm) are chosen. The results are obtained in the range of 0 to 1,000, with a higher value representing more melanin or erythema [66].



(A) (B)

Figure 14 (A) Mexameter and (B) Mexameter's probe (Model MX 18, Courage and Khazaka Electronic GmbH, Cologne, Germany).