CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

One of the approaches for increasing the skin penetration of drugs and many cosmetic chemicals is the use of vesicular systems, such as liposomes and niosomes. Conventional liposomes are usually not efficient as transdermally delivery systems across the skin, because they do not deeply penetrate the skin, but rather remain on the upper layer of stratum corneum (SC). Several researchers have developed novel elastic vesicles in order to deeply and easily penetrate through the skin. Ethanol, bile salts and many surfactants have been used to prepare these elastic vesicles. Ethanol is known as an efficient permeation enhancer and has been added in the vesicular systems to prepare the elastic vesicles. It can interact with the polar head group region of the lipid molecules, resulting in the reduction of the melting point of the SC lipid, thereby increasing lipid fluidity, and cell membrane permeability. The high flexibility of vesicular membranes from the added ethanol permits the elastic vesicles to squeeze themselves through the pores which are much smaller than their diameters. Thus, elastic vesicles could overcome the limitation of low penetration ability of the conventional liposomes or compounds in the commercial formulations across the skin. Ethosomes are vesicular systems composed mainly of phospholipids together with the high content of ethanol (20–45%). However, ethosomes have problems of variable purity and high cost of phospholipids. The more advantages promising novel

carriers for transdermal delivery of various drugs and cosmetic substances are the elastic vesicles composing of non-ionic surfactants (Tween 61 and Span 60).

The increase in the average age of the people, especially in Europe, North America and some other countries, which can expect to reach the changes in the lifestyles, have resulted in a quest for anti-aging products. The progressive increase in the aged populations has become the greatest burden of the health systems of the industrialized countries. As Americans are living longer, one manifestation of the obsession is that in 2005, the U.S. cosmeceutical market was estimated to be $12.5 billion, with the potential to exceed $16 billion by 2010. Hundreds of products are marketed to diminish fine lines and wrinkles, decrease redness, smooth texture, fade discoloration, and give a more youthful appearance to the skin. Nowadays, trends of anti-aging market value are increasing continuously. Alternative medicines, such as herbs appear to be more interesting than drugs or hormones which usually cause side effects. Recently, phytocosmetics have been popularly investigated. Thai medicinal plants have been claimed for anti-aging activity such as White Kaow Krua (*Pueraria mirifica* Airy Shaw et Suvatab), Asiatic pennywort (*Centella asiatica* L.) as well as many Lanna medicinal plants. As known, medicinal plants have many problems. For examples, it is not convenient to use, and most bioactive compounds in the plants are not chemically stable and also have problems of microbiological contamination during storage even kept in a refrigerator. Many technologies have been used to solve these problems, such as extraction and purification of the bioactive compounds, as well as using nanotechnology. In this present study, the Lanna medicinal plants which show an *in vitro* anti-aging activity was selected to prepare the semi-purified extract and developed to an elastic nanovesicular product for topical anti-aging application.

1.2 Objectives

The purpose of this study is to develop elastic nanovesicular formulations loaded with the semi-purified extracts containing bioactive compounds from Thai medicinal plants for topical anti-aging.

1.3 Scope of study

This study was divided into 3 parts which were the followings:

1.3.1 Extraction and purification and *in vitro* biological assay of anti-aging activity of the selected Thai plants.

1.3.1.1 Select at least 15 Thai medicinal plants from the Lanna medicinal plant recipe database which were most frequency used and had scientific evidences for anti-aging activity.

1.3.1.2 Prepare the crude extracts containing the bioactive compounds from at least 15 Thai medicinal plants obtained from the Lanna medicinal plant recipe database by solvent or aqueous extraction or sonication or by the traditional methods specified in the recipes.

1.3.1.3 *In vitro* biological anti-aging activity assay of the extracts from the selected 10 Thai medicinal plants, for examples, the DPPH, chelating, tyrosinase inhibition assay, cell proliferation activity or the inhibitory effect on the production of MMP-2 on human skin fibroblast. The phytochemical tests were also used to determine the groups of the compounds in the extracts.

1.3.1.4 Selection of the extracts containing the bioactive compounds which have the highest *in vitro* anti-aging activities. The extracts were fractionated, and the fractions were tested for *in vitro* anti-aging activities. The best fraction was selected and used to load in niosomes.

1.3.2 Prepare at least 3 elastic bilayer vesicular formulations (liposomes or niosomes) by the chloroform film method with sonication. Characteristics of the bilayer nanovesicular formulations were investigated according to the following procedures:

1.3.2.1. Determination of the particle size and lamellarity of the formulations by TEM.

1.3.2.2. Determination of the physical and chemical stability of the developed elastic niosomal formulations stored in transparent tubes covered with aluminum cap at 27 ± 2°C (room temperature), 45 ± 2°C and 4 ± 2°C for at least 3 months.

1.3.2.3. Determination of the deformability index of the vesicles by extrusion measurement.

1.3.2.4. Selection of the best elastic nanovesicular formulation.

1.3.3 The entrapment of the semi-purified extracts containing bioactive compounds in elastic niosomes were determined by gel filtration and HPLC assay, using a marker or a HPLC fingerprint.

1.3.4. Prepare at least 3 gel base formulations, select the best gel base and incorporate with the elastic niosomes loaded with the extracts containing the bioactive compounds and the following studies were investigated:

1.3.4.1 Determine the physical stability (layer separation, pH, viscosity and particle size) and chemical stability of the gel formulations containing elastic niosomes by keeping at room temperature (27 ± 2°C), 4 ± 2°C and 45 ± 2 °C for at least 3 months. Contents of the marker compounds were analyzed by HPLC.

1.3.4.2. Investigate rat skin permeation of the elastic niosomes loaded with the extract incorporated in gel by Franz diffusion cell.

1.3.4.3 Investigate skin irritation in rabbits by Draize skin test.

1.3.4.4. Investigate anti-aging activity of the developed gel formulations containing the extract with bioactive compounds loaded in elastic niosomes in human volunteers for anti-wrinkle or smoothness or hydration on the skin.

1.4 Literature reviews

The main disadvantage of transdermal drug delivery is the poor penetration of most compounds across the skin. The main barrier of the skin is the uppermost layer, the SC. Several approaches have been developed to weaken this skin barrier. One of the approaches for increasing the skin penetration of drugs and many cosmetic chemicals is the use of vesicular systems, such as liposomes and niosomes. Liposomes are unilamellar or multilamellar spheroid structures composed of lipid molecules, often phospholipids, assembled into bilayers (Bangham et al., 1965). However, liposomes have problems of variable purity and high cost of phospholipids. Niosomes or non-ionic surfactant based vesicles have been studied as an alternative to liposomes. Niosomes are formed from non-ionic surfactants in aqueous media resulting in closed bilayer structures (Florence and Baillie, 1989). In comparing to phospholipids vesicles (liposomes), niosomes offer higher chemical stability, lower costs, and great availability of surfactant classes (Handjani-Vila et al., 1979; Uchegbu and Florence, 1995; Uchegbu and Vyas, 1998). Drug delivery systems using vesicular systems such as liposomes (Betageri and Habib, 1994) or niosomes (Schreier and

Bouwstra, 1994) have advantages over conventional dosage forms because the vesicles can act as drug containing reservoirs. The modification of the vesicular compositions or surface properties can adjust the drug release rate and the affinity for the target site. Conventional liposomes and niosomes are usually not efficient to transdermally deliver across the skin, because they do not deeply penetrate the skin. But, they rather remain on the upper layer of SC. Several researchers have developed novel elastic nanovesicles in order to deeply and easily penetrate through the skin (Cevc, 1996; Touitou et al., 2000; Vaibhav et al., 2007). Ethanol, bile salts and many surfactants have been used to prepare these elastic vesicles. The high flexibility of vesicular membranes permits the elastic vesicles to squeeze themselves through the pores which are much smaller than their diameters (Van den Bergh, 1999; Manosroi et al., 2008). Thus, elastic nanovesicles could overcome the limitation of low penetration ability of the conventional liposomes and niosomes or compounds in the commercial formulations across the skin. Elastic nanovesicles have been successfully applied both in cosmeceuticals and pharmaceuticals.

1.4.1 Elastic nanovesicles

Elastic nanovesicles are novel types of liquid-state vesicles which have been developed in the early 1990s. Elastic nanovesicles, which composed of phospholipids, ethanol and water, could better penetrate the intact skin in comparing to the conventional vesicles since they can squeeze through small pores in SC which are smaller than their vesicular sizes and can also deliver the drugs or compounds of both low and high molecular weight. Furthermore, they can prolong the release and demonstrate a better biological activity in comparing to the conventional nanovesicles. Elastic nanovesicles are classified into phospholipid and detergent based types.

1.4.1.1 Classification of elastic nanovesicles

A. Phospholipid-based types

Phospholipid based elastic nanovescicles are the first generation of elastic vesicles, consisting of phospholipids and edge activators which are single chain surfactants such as cholate, Span 80 and Tween 80. Transfersome and ethosome are the examples of phospholipid based elastic nanovescicles.

- Transfersomes®: They are the phospholipids based elastic nanovesicles which are the first generation of elastic nanovesicles introduced by Cevc et al. (1996). Transfersomes are composed of phospholipids (PC) as their main ingredients with 10–25% of the edge activators. Sodium cholate, Span 80, Tween 80, oleic acid, and dipotassium glycyrrhizinate (KG) were employed as edge activators (El Maghraby et al., 2000; Trotta et al., 2002). **Table 1** summarizes the effect of Transfersomes® on transdermal drug delivery. Transfersomes® also have advantages on the cosmetic field. When applied on the intact skin, Transfersomes® are not detrimental to skin. Phospholipid, as a component of Transfersomes®, seems to improve aged skin hydration (Cevc and Blume, 2001). For drug delivery, Transfersomes® advantages include a faster onset of drug effect, longer times of action, a biological action that is unaffected by mechanical abrasion, and the ability to reduce the dosage needed to achieve therapeutic effects.

Table 1 Drugs skin penetration from Transfersomes® (Choi and Maibach, 2005)

Drugs (comments)	Animal	Composition
Dipotassium glycyrrhizinate (KG)	Pig	PC:KG (4:1)
		HPC:KG (4:1)
Methotrexate	Pig	PC:KG (2:1)
		HPC:KG (2:1)
Dexamethasone	Rat	PC:CHOL (7:3)
		PC:deoxycholate (85:15)
		PC:Tween-80 (85:15)
		PC:Span-80 (85:15)
Diclofenac	Rat	Commercial form
		Lotion-like transfersomes
Gap junction protein (antibody production)	Mouse	Soybean PC
		PC/sodium cholate/SDS
Insulin (decrease of blood glucose)	Mouse	PC liposomes or micelle
		PC/cholate (8.7:1.3)
Cyclosporin A	Mouse	PC/cholate (10:2.8)
Oestradiol	Human	PC/cholate (84:16)
		PC/Span 80 (84:16)
		PC/Tween 80 (84:16)
		PC/oleic acid (84:16)
5-Fluorouracil	Human	PC/cholate (84:16)
Rotigotine	Human	L-595/PEG-8-L (50:50)
Pergolide	Human	L-595/PEG-8-L (50:50)

- Ethosomes: They are phospholipid-based elastic nanovesicles containing high content of ethanol (20–45%). Ethanol is known as an efficient permeation enhancer and has been added in the vesicular systems to prepare the elastic nanovesicles. It can interact with the polar head group region of the lipid molecules, resulting in the reduction of the melting point of the SC lipid, thereby increasing lipid fluidity, and cell membrane permeability. The high flexibility of vesicular membranes from the added ethanol permits the elastic vesicles to squeeze themselves through the pores which are much smaller than their diameters (Van den Bergh, 1999; Touitou et al., 2000). Ethosomal systems are much more efficient in delivering substances to the skin in the terms of quantity and depth, than either

conventional liposomes or hydroalcoholic solution (Touitou et al., 2000). However, ethosomes may have problems of variable purity and high cost of the phospholipids. The ability of ethosomes to deliver minoxidil to the deep skin was investigated. Skin permeation was 45 and 35 times higher from the ethosomal system than 30% ethanolic solution and absolute ethanol, respectively. Similar results were obtained using acyclovir, testosterone, cannabidiol and ionic molecules such as propranolol and trihexylphenidyl (Touitou et al., 2001; Lodzki et al., 2003). The skin depth penetration from bacitracin ethosomes *in vivo* in rat evaluated after 8 h topical application to rat abdomen, bacitracin penetrated more deeply into skin from ethosomes than 30% ethanolic solution and liposomes (Godin and Touitou, 2004).

B. Detergent-based types

Detergent-based elastic nanovescicles are the second generation of the deformable or elastic vesicles. These vesicles which mainly consist of non-ionic surfactants are self forming bilayer vesicles in an aqueous solution and have high elasticity resulting from the solubility property of the surfactant(s). The first detergent-based elastic nanovescicles developed by Van den Bergh et al. (1999), consist of bilayer forming surfactant L-595 (sucrose laurate ester) and micelle-forming surfactant PEG-8-L (octaoxyethylene laurate ester). Manosroi et al. (2008) have developed the novel elastic niosomes composing of non-ionic surfactant (Tween 61), cholesterol, ethanol and water. The result has not only demonstrated the enhancement of transdermal absorption through rat skin, but also the *in vivo* anti-inflammatory effect of diclofenac diethylammonium when loaded in the developed novel elastic Tween 61 niosomes. Successful topical delivery of low molecular weight heparin was reported after incorporation into surface charged flexible vesicles made

of lipids with Tween 80. These vesicles were termed flexosomes and the cationic structures were the most efficient (Song and Kim, 2006). The Span 80-based ultra deformable vesicles, initially optimized by El Maghraby et al. (2000), have been recently employed for topical immunization.

1.4.1.2 Applications in topical pharmaceuticals

A. A depot formulation for pharmaceuticals

Twinkal et al. (2008) have developed the topical dosage form of an anti-migraine agent (rizatriptan) which can selectively deliver the drug to carnial nerves in the brain and ear. The *in vitro* skin permeation across rat skin of rizatriptan loaded in elastic liposomal formulations was proximately 8–19 times higher than the drug in solution. The amount of drug deposited and the biological activity of the drug in an optimized elastic liposomal formulation was 10 and 3 folds higher than the drug in solution, respectively. This result has indicated that elastic liposomal formulation can provide a sustained action of the drugs due to the depot effect in the deeper layer of the skin.

B. Improvement of transdermal delivery of the drug

Elsayed et al. (2006) have demonstrated that the drugs inside the Transfersomes® showed significant improvement in cumulative amount permeated and skin deposited after 24 h over the drug in an aqueous solution. They have suggested that both the penetration enhancing effect and the intact vesicle permeation into the SC might play an important role in improving skin delivery of the drugs by Transfersomes® under the non-occlusive conditions. Transfersome® can also be applied to transcutaneous immunization which is a novel needle-free immunization method developed for vaccines (Elsayed et al., 2006; Twinkal et al., 2008). Dinesh et

al. (2006) have investigated the transdermal potential of ethosomes bearing methotraxate (MTX), an anti-psoriatic and anti-neoplastic which is highly hydrosoluble with limited transdermal permeation. The formulation containing 3% phospholipid and 45% ethanol showed the highest entrapment, optimal nanometric size range, very low aggregation and growth in vesicular size after 120 days of storage. MTX loaded ethosomal carriers also provided an enhanced transdermal flux of 52.7 ± 4.34 μg/cm^2/h and decreased lag time of 0.9 h across human cadaver skin. This formulation has also demonstrated an enhanced permeation of rhodamine red to the deeper layers of the skin (170 μm) and retained its penetration power after storage. Elastic niosomes can be modified to have cationic characteristics by adding cationic lipids, such as dimethyl dioctadecyl ammonium bromide (DDAB), in the vesicular compositions. Elastic cationic niosomes are useful to deliver genetic materials in gene therapy. The positive charge on the vesicular surface can interact with the negative charge of DNA or any genetic materials by electrostatic interaction. Manosroi et al. (2009) have demonstrated the 100% entrapment efficiency of luciferase plasmid in elastic cationic niosomes with higher pLuc stability than in non-elastic cationic niosomes. For transdermal absorption, the average fluxes of pLuc loaded in elastic niosomes in viable epidermis and receiving solution at 6 h were 2.84 ± 0.04 and 1.96 ± 0.21, respectively (**Table 2**), whereas no pLuc was found in the receiving solution for the unloaded and loaded pLuc in non-elastic niosomes. This is certainly the effects of ethanol existing in the vesicles. Various mechanisms have been reported for the skin permeation enhancement effect of ethanol, for examples, by increasing the diffusion of the drugs through the lipid pathway of the skin (Hatanaka et al., 1993), reduction of lipid polar head interactions or disordering liquid-crystalline phases

within the membrane (Knutson et al., 1990), and increasing the drug solubility in the SC (Megrab et al., 1995). Furthermore, the surfactant compositions in the niosomal formulation can also change the structure of the SC resulting from their solubilization property.

C. Enhancement of biological activities

Gupta et al. (2005) have demonstrated that Transfersomes® containing soya phosphatidylcholine (SPC)/sodium deoxycholate (SDC) at 85:15% w/w showed the highest entrapment efficiency of tetanus toxoid (72.7 ± 3.4) and deformability index (124 ± 4.2) with the optimum vesicular size (196 ± 10.2 nm) in comparing to the conventional liposomes and niosomes. For topical immunization in albino rats, the maximum response of Transfersomes® loaded with tetanus toxoid was observed after 42 days. After secondary immunization on day 28, Transfersomes® elicited the maximum immune response again on day 42. The response was significantly comparable to that elicited by intramuscular injection of the same dose of alum-adsorbed tetanus toxoid. In comparison to Transfersomes®, liposomes and niosomes elicited weaker immune response. Thus, Transfersomes® are promising effective non-invasive topical delivery systems for antigens. Triterpene saponins, such as ammonium glycyrrhizinate, which have an anti-inflammatory activity, can be applied as a potential topical anti-inflammatory drug by using certain drug delivery systems such as ethosomes. Paolino et al. (2005) have demonstrated that ethosomes composed of ethanol 45% (v/v) and lecithin 2% (w/v) elicited an increase *in vitro* percutaneous permeation of ammonium glycyrrhizinate (63.2% of the applied dose). Ethosomes showed good skin tolerability in human volunteers when applied for a long period (48 h) and are able to significantly enhance the anti-inflammatory activity

Table 2 The cumulative amounts (μg/cm^2) and fluxes (μg/cm^2/h) of pLuc in SC (stratum corneum), VED (whole skin of viable epidermis and dermis), and receiver chamber following transdermal absorption across excised rat skin by vertical Franz diffusion cells at 6 hours (Manosroi et al., 2009)

	Cumulative amounts of pLuc (μg/cm2)			Fluxes (μg/cm2/h)		
Methods	SC	VED	Receiver chamber	SC	VED	Receiver chamber
Free pLuc	0	0	0	0	0	0
Free pLuc (SC stripping)	0	0	0	0	0	0
Free pLuc (iontophoresis)	0	0	0	0	0	0
Nonelastic liposomes	0	0	0	0	0	0
Nonelastic liposomes (SC stripping)	0	4.37 ± 0.74	0	0	2.73 ± 0.46	0
Nonelastic liposomes (iontophoresis)	0	2.80 ± 0.49	4.02 ± 0.19	0	7.01 ± 1.22	6.71 ± 0.31
Nonelastic niosomes	0	0	0	0	0	0
Nonelastic niosomes (SC stripping)	0	2.70 ± 0.52	0	0	3.83 ± 0.73	0
Nonelastic niosomes (iontophoresis)	0	3.84 ± 0.53	4.60 ± 0.15	0	9.60 ± 1.31	8.82 ± 0.28
Elastic liposomes	0	3.72 ± 0.11	2.04 ± 0.06	0	2.79 ± 0.09	1.92 ± 0.10
Elastic niosomes	0	3.79 ± 0.05	2.07 ± 0.03	0	2.84 ± 0.04	1.96 ± 0.21

Values represent as the mean ± standard deviation (n = 3).

and the sustained release of ammonium glycyrrhizinate in comparing to an ethanolic or aqueous solution.

1.4.1.3 Applications in cosmetics

The advantages of applying nanovesicles in cosmetics and cosmeceuticals are not only to increase the stability of the cosmetic chemicals and decrease of the skin irritation for those irritating chemicals, but also to enhance the transdermal permeation, especially for the elastic nanovesicles. However, the compositions and sizes of the vesicles are the main factors to be considered to obtain these advantages of elastic vesicles for cosmeceutical applications. Topical administration of many antioxidants is one of several approaches to diminish oxidative injury in the skin for cosmetic and cosmeceutical applications. But, antioxidants are usually not stable and can be degraded by exposing to light. These antioxidants include vitamin E, vitamin C, and flavonoids. Vitamin E is one of the major exogenous lipophilic antioxidants which are usually found in tissues. Its topical application can enhance the skin protection from exogenous oxidants. When vitamin E is added to cosmetic and many dermatological products, it is found to decrease the production of lipid peroxides in the epidermis as well as to protect against UV exposure (Record et al., 1991; Wissing and Muller, 2001) and those destructive chemicals and physical agents (Weber et al., 1997). In order to deliver vitamin E into the deeper layer of SC, Marina et al. (2006) have formulated several deformable liposomes by using hydrogenated soya lecithin (HPC) and sodium cholate (SCL), polysorbate 80 (T80), dipotassium glycyrrhizinate (DPG), or saccharose monopalmitate (SMP). The lipid to surfactant in w/w ratio which is necessary to obtain elastic vesicles depends on the O/W surfactant and ranges from 4:1 to 20:1.

Deformability of the elastic liposomes was confirmed by filtration through the microporous filters and differential scanning calorimetry measurements. For *in vitro* permeation studies, all systems showed negligible fluxes, below the UV-HPLC detection limit (**Table 3**). This has suggested that although elastic and non-elastic liposomes are not beneficial for delivery of α-tocopherol through the skin, the entrapment of the vitamin either in elastic or non-elastic liposomes can increase its photo-stability under UVB irradiation (**Figure 1**).

Table 3 α-Tocopherol skin permeation and skin accumulation from different liposomes containing 0.17% α-tocopherol and from a control solution. (T80 = Polysorbate 80, SCL = sodium cholate, HPC = hydrogenated soya lecithin, DPG = dipotassium glycyrrhizinate and SMP = saccharose monopalmitate (Marina et al., 2006)

System	Flux	Deformability	α-Tocopherol in the skin ($\mu g\ cm^{-2}$)
HPC	Negligible	No	3.5 (0.9)
6 : 1 HPC-DPG	Negligible	No	7.6 (0.4)
2 : 1 HPC-DPG	Negligible	No	21.6 (1.3)
6.25 : 1 HPC-DPG	Negligible	No	3.8 (0.5)
6.25 : 1 HPC-SCL	Negligible	Yes	27.0 (3.8)
6.25 : 1 HPC-T80	Negligible	Yes	29.2 (2.5)
6.25 : 1 HPC-SMP	Negligible	Yes	33.3 (2.9)

Values in parentheses are standard deviations.

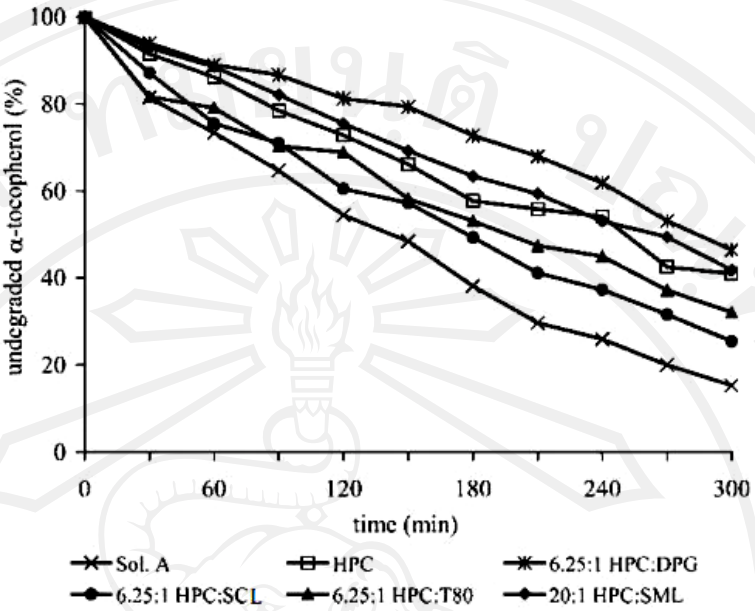


Figure 1 UVB photodegradation profiles of 0.17% w/w α-tocopherol in HPC and HPC with surfactant liposomes at various molar ratio (T80 = Polysorbate 80, SCL = sodium cholate, HPC = hydrogenated soya lecithin, DPG = dipotassium glycyrrhizinate and SMP = saccharose monopalmitate (Marina et al., 2006)

1.4.2 Transdermal delivery systems

Transdermal drug delivery systems for both local and systemic effects provide a convenient route of administration for a variety of clinical indications. However, the application of transdermal delivery is limited by the barrier function of the skin (Benson, 2005). In order to overcome this problem and optimize the transdermal delivery systems, it is necessary to understand the structure of the skin and pathways of skin penetration.

1.4.2.1 Skin structure and route of skin penetration

The basic skin structure is depicted in **Figure 2**. The main layers are the stratum corneum (SC), the viable epidermis including the basal membrane, the

dermis and the subcutaneous fat. Local skin structures are blood vessels, hair follicles, nerves, sebaceous glands and the sweat glands (Briggaman and Wheeler, 1975).

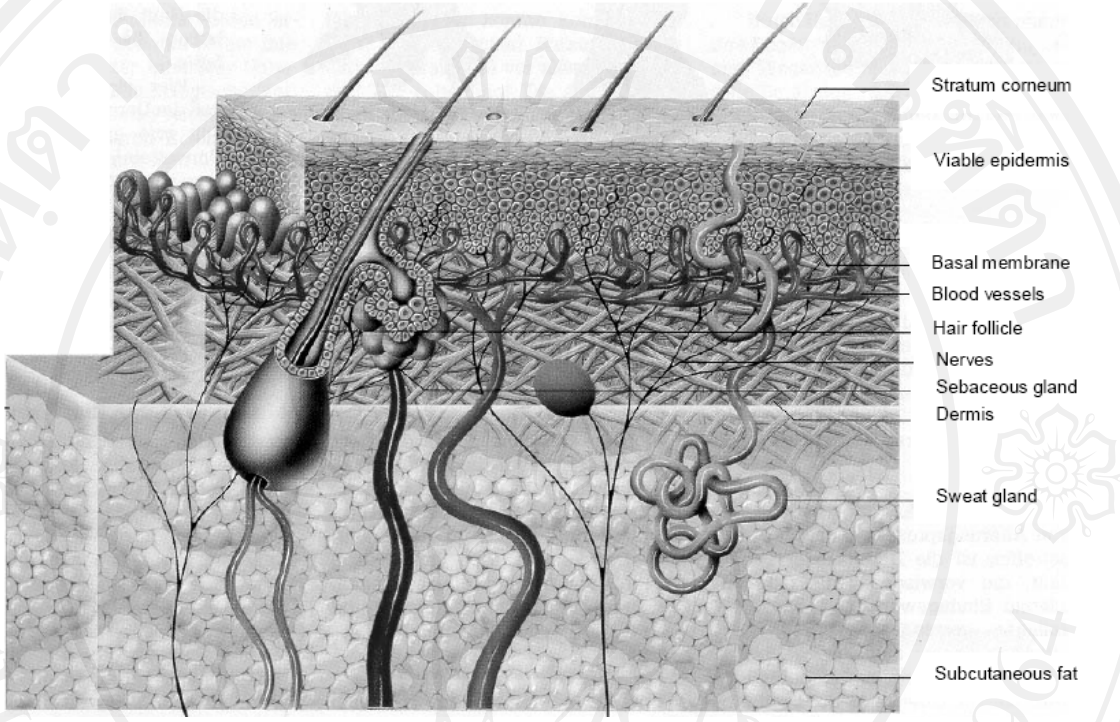


Figure 2 Skin structure of human skin (Briggaman and Wheeler, 1975)

SC is highly hydrophobic and contains 10–15 layers of the interdigitated corneocytes (thickness of 10–20 μm). Its organization can be described by the 'brick and mortar' model (Elias, 1983). Due to its highly organized structure, SC is the major permeability barrier to external materials. It is regarded as the rate-limiting factor in the penetration of therapeutic agents through the skin.

Viable epidermis consists of multiple layers of keratinocytes at various stages of differentiation. The basal layer contains actively dividing cells, which migrate upwards to successively form the spinous, granular and clear layers. The role of the viable epidermis in skin barrier function is mainly related to the intercellular lipid

channels and to several partitioning phenomena. Depending on their solubility, drugs can partition from layer to layer after diffusing through the SC.

Dermis and hypodermis is largely acellular. But, it is rich in blood vessels, lymphatic vessels and nerve endings. The elasticity of the dermis is attributed to a network of protein fibers, including collagen (type I and III) and elastin, which are embedded in an amorphous glycosaminoglycan ground substance. The dermis also contains scattered fibroblasts, macrophages, mast cells, leukocytes, hair follicles, sebaceous and sweat glands.

When drugs or cosmetics are applied on the skin surface, penetration into and through the skin can occur via various routes. Substances penetrate either via the SC (transepidermal) or via the appendages (transappendageal) (**Figure 3**) (Moser et al., 2001). During penetration through the SC, two possible routes can be distinguished which are: (i) penetration alternating through the corneocytes and the lipid lamellae (transcellular route) and (ii) penetration along the tortuous pathway along the lipid lamellae (intercellular route). Generally, it is accepted that the predominant route of penetration through the SC is the intercellular route. This is mainly caused by the densely cross-linked cornified envelope coating the keratinocytes. However transcellular transport for small hydrophilic molecules such as water can not completely be excluded (Schaefer and Redelmeier, 1996). The appendageal route or shunt route includes either the duct of the sweat glands or the follicular duct. The content of sweat glands is mainly hydrophilic, while the content of the follicular duct is lipophilic. This is mainly due to the sebum excreted into the opening of the follicular duct. It is generally accepted that due to its large surface area, passive skin permeation mainly occurs through the intact SC. Since the appendages cover

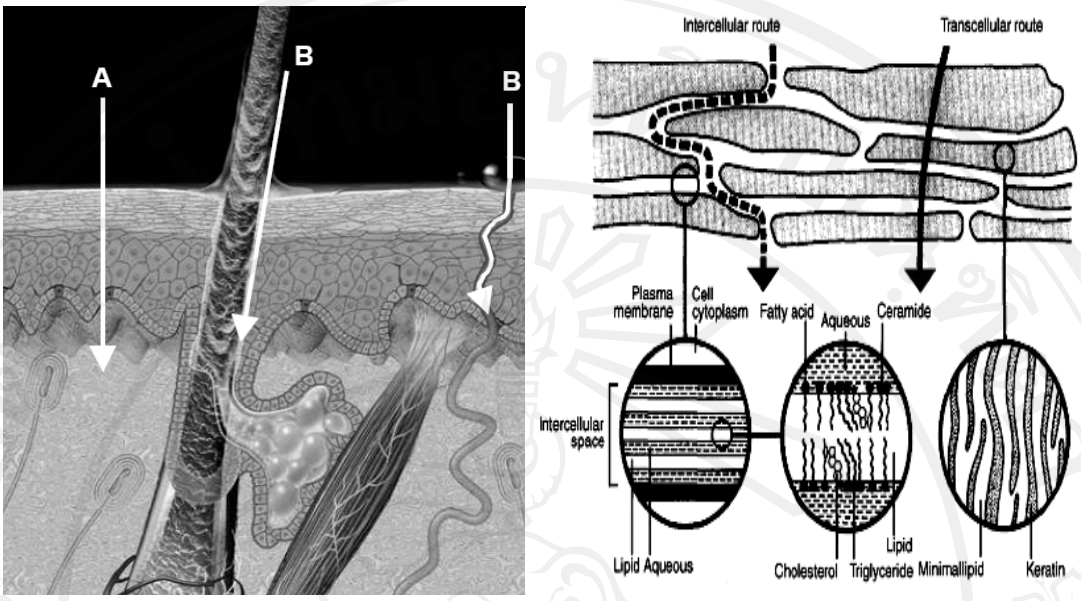


Figure 3 Two main pathways of skin penetration including transepidermal route (A), transappendageal route including hair follicles with their associated sebaceous glands (B) (left) and transepidermal route containing two micro-pathways including intercellular and transcellular pathway (right) (Barry, 2001)

0.1 % of the total skin surface, it is discussed that the permeation along appendages contributes only slightly to the overall passive transdermal penetration (Keister and Kasting, 1986).

Advantages of transdermal delivery systems

The advantages of transdermal delivery have been well documented (Guy and Hadgraft, 1989). Their therapeutic benefits are the sustained delivery of drugs (to provide a steady plasma profile, particularly for drugs with short half-lives, and hence reduced the systemic side effects); the reduction of the typical dosing schedule to once daily or even once weekly, hence generating the potential for the

improved patient compliance and the avoidance of the first-pass metabolism effect for drugs with poor oral bioavailability. Additionally, transdermal delivery represents a convenient, patient-friendly option for drug delivery with the potential for flexibility, easily allowing dose changes according to patient needs and the capacity for self-regulation of dosing by the patients. Alternatively, transdermal delivery can be used in the situations requiring the minimal patient cooperation, for examples, in situations involving administration of drugs by someone other than the patients. The non-invasive character of transdermal delivery makes it accessible to a wide range of patient populations and a highly acceptable option for drug dosing. Nowadays, eight drugs based on transdermal delivery system are currently in the market including clonidine, estradiol, nitroglycerine, fentanyl, testosterone, scopolamine, nicotine and oxybutinin (Farahmand et al., 2009). In the cosmetic aspects, transdermal delivery systems have been claimed for the delivery of active ingredients to the site of action (SC or viable skin layers) for the sufficient long period of times (Lasic, 1995, Magdassi, 1997). Since about 20 years after the introduction of liposomes to the cosmetic market, similar innovative novel nanocarriers such as solid lipid nanoparticles (SLN) and nanostructure lipid carriers (NLC) have been established as the second generation products after liposomes.

1.4.2.2 Skin penetration enhancement

Several skin penetration enhancement techniques have been proposed to improve bioavailability and increase the range of drugs for topical and transdermal delivery. **Figure 4** summarizes the techniques to optimize transdermal drug delivery (Benson, 2005).

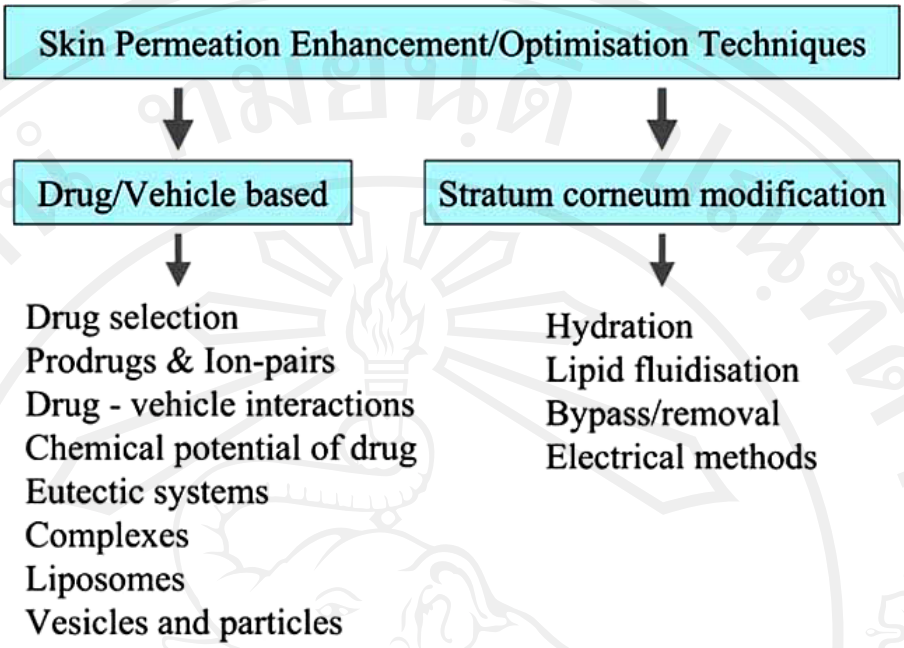


Figure 4 Techniques to optimize drug permeation across the skin (Benson, 2005)

A. Drug/vehicle based

A.1 Drug selection

The simplest technique for drug selection is to choose a drug from pharmacological class with the suitable physicochemical properties to translocate across the barrier at an acceptable rate. The ideal properties of molecule penetrating SC are: (i) low molecular mass, preferably less than 500 Da, and the diffusion coefficient tends to be high; (ii) adequate solubility in oil and water that the skin concentration gradient may be high (the driving force for diffusion); (iii) optimal partition coefficient and (iv) low melting point correlating with good solubility (Benson, 2005) such as caffeine, corticosterone and dexamethsone (Mitragotri et al., 1995; Johnson et al., 1996).

A.2 Prodrugs and ion-pairs

The prodrug approach has been investigated to enhance dermal and transdermal delivery of drugs with unfavorable partition coefficients. The prodrug design strategy generally involves the addition of a promoiety to increase partition coefficient and hence solubility and transport of the parent drug in the SC. Upon reaching the viable epidermis, esterases release the parent drug by hydrolysis, thereby optimising solubility in the aqueous epidermis. Charged drug molecules do not readily partition into or permeate through human skin. Formation of lipophilic ion-pairs has been investigated to increase SC penetration of the charged species. This strategy involves adding an oppositely charged species to the charged drug, forming an ion-pair in which the charges are neutralized. So, the complex can partition into and permeate through the SC. The ion-pair then dissociates in the aqueous viable epidermis releasing the parent charged drug which can diffuse within the epidermal and dermal tissues (Benson, 2005). The intrinsic poor permeability of the very polar 6-mercaptopurine was increased up to 240 times using S^6-acyloxymethyl and 9-dialkylaminomethyl promoieties (Saab et al., 1990) and that of 5-fluorouracil, a polar drug with reasonable skin permeability was increased up to 25 times by forming *N*-acyl derivatives (Beall and Sloan, 2001).

A.3 Chemical potential of drugs in the vehicle

The maximum skin penetration rate is obtained when a drug is at its highest thermodynamic activity as is the case in a supersaturated solution. Supersaturated solutions can occur due to the evaporation of the solvent or by mixing of the co-solvents. Clinically, the most common mechanism is the evaporation of solvent from the warm skin surface which probably occurs in many topically applied

formulations. In addition, if water is imbibed from the skin into the vehicle and acts as an antisolvent, the thermodynamic activity of the permeation would increase (Kemken et al., 1992). Increases in drug flux of five- to ten-fold have been reported from supersaturated solutions of a number of drugs including hydrocortisone acetate, piroxicam and ibuprofen (Davis and Hadgraft, 1991; Pellett et al., 1994; Iervolino et al., 2000).

A.4 Eutectic systems

The melting point of a drug influences solubility and hence skin penetration. Lower the melting point exhibit greater the solubility of a material in a given solvent, including skin lipids. The melting point of a drug delivery system can be lowered by formation of a eutectic mixture: a mixture of two components which, at a certain ratio, inhibit the crystalline process of each other, such that the melting point of the two components in the mixture is less than that of each component alone. Eutectic mixture of lidocain and menthol system has promoted permeation through the snake skin (Kang et al., 2000). Stott et al. (2001) have shown that eutectic mixture of ibuprofen with seven terpenes and propanol with fatty acid enhances the transdermal drug permeation.

A.5 Complexes

Complexation of drugs with cyclodextrins has been used to enhance aqueous solubility and drug stability. Cyclodextrins of pharmaceutical relevance contain 6, 7 or 8 dextrose molecules (α-, γ-, β-cyclodextrin, respectively) bound in a 1, 4-configuration to form rings of various diameters. The ring has a hydrophilic exterior and lipophilic core in which appropriately sized organic molecules can form

non-covalent inclusion complexes resulting in an increased of aqueous solubility and chemical stability. Derivatives of β-cyclodextrin with increased water solubility (e.g. hydroxypropyl-β-cyclodextrin HP-β-CD) are most commonly used in pharmaceutical formulation. Cyclodextrin complexes have been shown to increase the stability, wettability and dissolution of the lipophilic insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET) (Szente et al., 1990) and the stability and photostability of sunscreens (Scalia et al., 1999).

A.6 Liposomes and other vesicles

There are many examples of cosmetic products in which the active ingredients are encapsulated in liposomes including ascorbic acid and α-tocopherol. These include humectants such as glycerol and urea, sunscreening, tanning agents, enzymes, elastin polypeptides and *Aloe vera*. Several hundred cosmetic products are commercially available since Capture (C. Dior) and Niosomes (L'Or´eal) were introduced in 1987. These cosmetic products range from simple liposome pastes which are used as a replacement for creams, gels, and ointments for do-it-yourself cosmetical products to formulations containing various extracts, moisturizers, antibiotics, and to complex products containing recombinant proteins for wound or sunburn healing. Most of the products are anti-aging skin creams, sunscreens, long lasting perfumes and hair conditioners (Hong and Park, 1999; Sinico et al., 2005). Although there are few commercial topical products containing encapsulated drugs, there are considerable researches in the topic. A variety of encapsulating systems have been evaluated including liposomes, deformable liposomes or transfersomes, ethosomes and niosomes. The potential of liposomes for delivering drugs to the skin was first reported by Mezei and Gulasekharam (1980). They have demonstrated that

the skin delivery of triamcinolone acetonide was four to five times greater from a liposomal lotion than an ointment containing the same drug concentration (Mezei and Gulasekharam, 1980). Their delivery mechanism is reported to be associated with the accumulation of liposomes and the associated drug in the SC and upper skin layers, with minimal drug penetrating to the deeper tissues and systemic circulation. The mechanism of the enhanced drug uptake by liposomes into the SC is unclear. It is possible that the liposomes either penetrate the SC to some extent then interact with the skin lipids to release their drug or that only their components enter the stratum corneum. It is interesting that the most effective liposomes are reported to be those composed of lipids similar to SC lipids, which are likely to most readily enter SC lipid lamellae and fuse with endogenous lipids.

B Stratum corneum (SC) modification

B.1 Hydration

Normally, SC contains 15–20% water. When SC is hydrated, the water percentages can be increased up to 50%. This situation can directly affect the permeability of the skin to the substances. It has been suggested that the increasing skin hydration may increase the absorption of all substances penetrating the skin. Therefore, the occlusive films, hydrophobic ointments and transdermal patch are used to enhance drug bioavailability into the skin (Barry, 2001). Occlusive films of plastic or oily vehicle have the most effect on hydration and penetration rate. A commercial example of this is the use of an occlusive dressing to enhance skin penetration of lignocaine and prilocane from cream in order to provide sufficient local anaesthesia within about 1 h. Also drug delivery from many transdermal patches benefits from occlusion (Roberts and Walker, 1993; Wester and Maibach, 1995).

B.2 Lipid disruption/fluidisation

Many enhancers, such as azone, DMSO, alcohols, fatty acids and terpenes, have been shown to increase permeability by disordering or 'fluidising' the lipid structure of the SC. The diffusion coefficient of a drug is increased as the enhancer molecules form microcavities within the lipid bilayers, hence increasing the free volume fraction. In some cases, the enhancers penetrate into and mix homogeneously with the lipids. However, others such as oleic acid and terpenes, particularly at high concentration, pool within the lipid domains to create permeable 'pores' that provide less resistance for polar molecules. These effects have been demonstrated using differential scanning calorimetry (DSC) to measure the phase transition temperature, electron spin resonance (ESR) studies, fourier transform infrared (FTIR), Raman spectroscopy and X-ray diffractometry. These enhancer compounds consist of a polar head group with a long alkyl chain and are more effective for hydrophilic permeants, although an increased delivery of lipophilic permeants has also been reported (Bouwstra et al., 1989). A sample summary of enhancers includes: water, hydrocarbons, sulphoxides (especially DMSO) and alcohols, azone and its derivatives, surfactants (anionic, cationic and non-ionic), amides (including urea and its derivatives), polyols, essential oils, terpenes and derivatives, oxazolidines (Asbill et al., 2000; Sinha and Kaur, 2000). Reddy et al. (2000) concisely review antiselective permeation, with and without chiral enhancers, including terpenes. The effect of ionisation and enhancers on permeation through skin and silastic has been considered (Smith and Irwin, 2000).

B.3 Bypass or removal of SC layer

The pilosebaceous unit (hair follicle, hair shaft and sebaceous gland) provides a route that bypasses the intact SC. It also represents a drug delivery target for many substances such as topical 'naked' DNA for gene therapy (Hoffman, 2000). However, only molecule with the size of 3–10 μm can be concentrated in the follicle. The penetration through the follicles was reached when the molecular size is less than 3 μm (Schaefer and Redelmeier, 1996). Several skin bypassed or removed based devices have been used to increase drug penetration, for examples, microneedle array is a device of 400 microneedles which insert drug just below the SC barrier. The penetration rate increases up to 100,000 folds without breaking or stimulating nerves in deeper tissue by this technique (McAllister et al., 2000; Asbill et al., 2000). Besides microneedle, microdermabrasion (a stream of aluminum oxide crystal), dermabrasion (a motor-driven abrasion) and adhesive tapes are also extended to enhance drug penetration through the skin (Friedland and Buchel, 2000).

B.4 Electrical method

Ultrasound (phonophoresis, sonophoresis, ultrasonic energy at low frequency) disturbs the lipid packing in SC by cavitation. This technique is used for transdermal delivery of large polar molecules such as phonophoresis of insulin, erythropoietin and interferon (Mitragotri et al., 1995).

Iontophoresis, the electrical driving of charged molecules into tissue, passes a small direct current (approximately, 0.5 mA/cm^2) through a drug-containing electrode in contact with the skin. This technique is considerably interested in transdermal delivery of therapeutic peptides, proteins and oligonucleotides (Chiang et al., 1998; Brand et al., 1998).

Skin electroporation creates transient aqueous pores in the liquid bilayers by the application of short (micro- to millisecond) electrical pulses of approximately 100–1000 V/cm. The skin penetration rate increased 10 to 10^4 folds for neutral and highly charged molecules of up to 40 kDa (Chang et al., 2000). The process may also transport into the integument, vaccines, liposomes, as well as nanoparticles and microspheres (Prausnitz et al., 1996). Electroporation may combine with iontophoresis to enhance the penetration of peptides such as vasopressin, neurotensin, calcitonin and LHRH (Riviere et al., 1995; Banga et al., 1999).

Magnetophoresis uses magnetic fields to move diamagnetic materials through skin. This technique delivers drugs in a controlled and pusatile mode (Santini et al., 1999). The static magnetic field could facilitate the transdermal permeation of drugs such as benzoic acid, salbutamol sulfate and terbutaline sulfate (Murthy, 1999; Murthy and Hiremath, 1999; Murthy and Hiremath, 2000).

Photomechanical wave, the laser pulse irradiates on a drug solution which is placed on the skin and covered by black polystyrene. The resulting photomechanical wave stresses SC and enhances drug delivery (Lee et al., 1999). It has been shown to induce a transient increase of the permeability of the plasma membrane of cells *in vitro* without affecting cell viability (Flotte et al., 1995). In addition, it has been shown that a photomechanical wave renders the SC more permeable to small molecules which was aminolevulenic acid (Doukas et al., 1995).

1.4.2.3 Interaction of elastic nanovesicles with skin

Elastic nanovesicle and skin interactions can occur either at the skin surface or in the deeper layers of the SC. Elastic nanovesicle and skin interactions are strongly influenced by the composition of the vesicles, resulting in the differences in

their phase state and elasticity. Cevc and Blume (1992) proposed Transfersomes® which are drug carrier systems that can penetrate across the intact skin. It is believed that the successful passage of such carriers is based on the two important factors. First, the highly elasticity (deformability) of the vesicle bilayers and second, the existence of an osmotic gradient across the skin. Because of high deformability, Transfersomes® could squeeze between the SC cells and carry the loaded drug across the intact skin. Fang et al. (2001) investigated the mechanism of vesicular system across the skin with soybean phosphatidylcholine (PC) liposomes containing enoxacin. After 12 h pretreatment, drug permeation across the PC treated skin was higher than that across the nontreated skin. These results indicated that the PC liposomes could serve as permeation enhancers for drug delivery. For 5-fluorouracil Transfersomes®, the percentage of drug penetrated (13.5%) was higher than the drug entrapment efficiency (8.8%) of Transfersomes® (Guo et al., 2000). This suggested that Transfersomes® components may have altered the skin structure, as penetration enhancers. Touitou et al. (2000) proposed an action mechanism of ethosomal system. First, ethanol disturbs the organization of the SC lipid bilayer and enhances its lipid fluidity. The flexible ethosomal vesicles can then penetrate the disturbed SC bilayers. The release of drug in the deep layers of the skin and its transdermal absorption could then be the result of the fusion of ethosomes with skin lipids and the drug release at various points along the penetration pathway. Unlike other elastic vesicles, occlusion slightly increased the skin penetration of ethosomes. For further mechanism investigation of ethosomal skin permeation, Godin and Touitou (2004) have used the double-staining methods including ethosomes co-loaded with the two fluorescent probes which were rhodamine red (RR) and FITC-bacitracin (FITC-Bac). Both probes

were delivered from ethosomes to a maximal possible depth of 200 µm. When the two probes were observed separately at the skin depth of 90 µm, it is clearly seen that the delivery of FITC-Bac from ethosomes was followed by the delivery of ethosomal

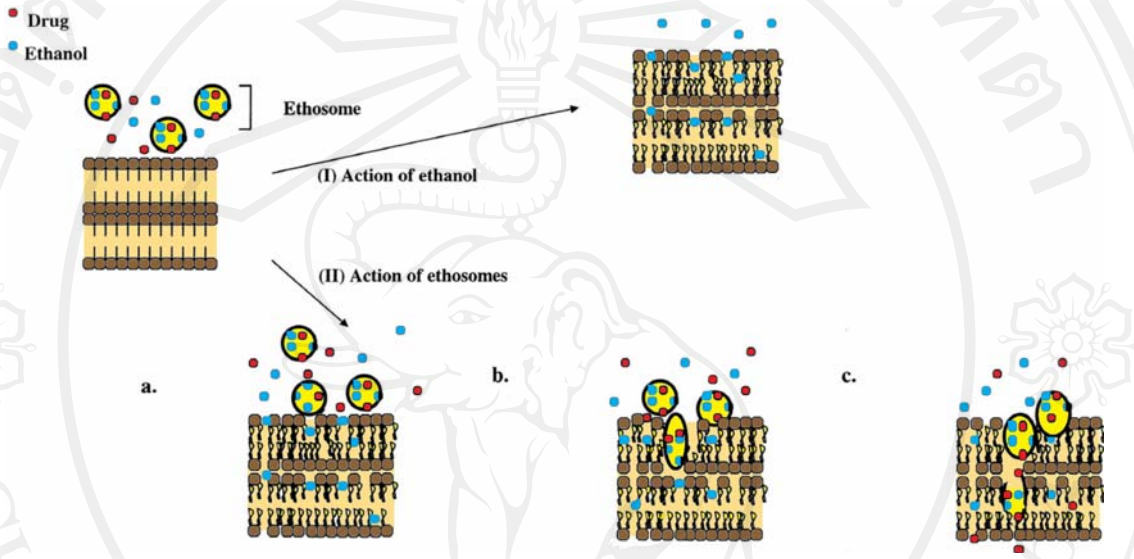


Figure 5 Mechanism to penetrate the skin of ethosomes (Touitou et al., 2000)

components in the same area. Skin penetration profile indicated that penetration of ethosomal vesicles into the skin peaked at 40 µm, while depth of the maximum bacitracin penetration was 90 µm, suggesting that the release of the drug in deep skin layers occurred. In a double staining study, bacitracin delivered from ethosomes by entering the skin between the corneocytes through the intercellular lipid domain. High contents of ethanol fluidize the ethosomal membranes to produce highly deformable vesicles. Subsequently, ethosomes squeeze drugs between the cells in the SC and carry large amounts of the drugs across the intact skin (Valjakka-Koskela et al., 1998; El Maghraby et al., 2001).

1.4.3 Thai Lanna medicinal plants database

1.4.3.1 Thai Lanna medicinal plant textbook database

Lanna included 7 provinces of the northern part in Thailand (Chiang Mai, Chiang Rai, Lamphun, Lampang, Phayao, Phrae and Nan). The Thai Lanna region used to be an independent country for 700 years ago. It has its own folklores and wisdoms in various fields, such as politics, agriculture and traditional medicines. The Lanna Thai medicinal recipes were recorded as Lanna scripts in palm leaves, mulberry paper or S*treblus asper* paper. Since 1997, Natural Products Research and Development Center (NPRDC) by Prof. Dr. Jiradej Manosroi has collected many Lanna plant recipes for various treatments. So far, NPRDC has collected and translated of about 21 Lanna medicinal textbooks from 7 provinces in the northern part of Thailand. These were typed by using Lann alphabet font (LN_TILOK_V1_4.OTF), translated from Lanna alphabet to Thai alphabet and understandable Thai language. All were recorded in the "Lanna Medicinal Plant Textbook Database: Recipes-Disease-Medicinal Plant" program named "Manosroi II" which has been developed by NPRDC. The computer program with the information was tested for the ability to search with keywords and print out the reports. The keywords included the specific name of the diseases, plants and recipes in Lanna Thai, modern Thai and English alphabets. The database contained 35,823 recipes with 3,614 diseases and 7,480 medicinal plants (updated on August 2, 2010). A number of health care recipes were also found. This database can be used to select many potential recipes for the primary health care and the research and development of drugs, cosmetics and food supplement. Many recipes implied the anti-aging applications. For examples, recipe number: Chiang Mai / 018-048 / 80 048 08 042-

042 included 3 Lanna plants (*Boesenbergia rotunda*, *Brassica chinensis* and *Acorus calamus*) have been used for skin nourishment. (Manosroi et al., 2006)

Figure 6 Lanna Medicinal Plant Textbook Database: Recipes-Disease-Medicinal Plant "Manosroi II" (Manosroi et al., 2006)

1.4.3.2 Plant selection

The medicinal plants can be selected from the Thai Lanna Medicinal Plant Textbook Database program version "Manosroi II" developed by NPRDC, Science and Technology Research Institute (STRI), Chiang Mai University in Thailand using the keywords which related to anti-aging applications such as anti-aging, longevity and skin nourishment. Thirty-one recipes have been found from the

database (**Table 4**). The frequent traditional use and scientific evidences for anti-aging and longevity indicated in the recipes can be used to select the plants (**Table 5**).

1.4.4 The 15 selected Thai Lanna medicinal plants

1.4.4.1 *Acorus gramineus* L. (**Figure 7**)

This plant has Thai common name of "Wannam". *A. gramineus* exhibits sedative, digestive, analgesic, diuretic, and antifungal actions. Anthelmintic and pesticidal activities of *A. gramineus* have been reported to be associated with the phenylpropanoids α- and β-asarones (Perrett and Whitfield, 1995). α- and β-Asarones, the major essential oil components in *A. gramineus*, exhibited neuroprotective action against the excitotoxicity induced by *N*-methyl-D-aspartate (NMDA) or glutamate (Glu) in cultured rat cortical cells (Cho et al., 2001). β-Asarone isolated from the rhizome of a related species, *Acorus calamus*, was shown to have anthelmintic and antibacterial activities against *Bacillus subtilis* and *Staphylococcus aureus* (McGaw et al., 2002).

Table 4 Sources and numbers of anti-aging Thai Lanna medicinal-plant recipes selected from the Thai Lanna Medicinal Plant Textbook Database program version "Manosroi II"

Province	Numbers of anti-aging recipes
Chiang Mai	2
Chiang Rai	12
Lampang	2
Lamphun	6
Phrae	3
Nan	5
Payao	1
Total	31

Table 5 The 15 selected Thai Lanna medicinal plants with their frequent use selected from the 31 Lanna medicinal-plant recipes

Scientific name	The frequency found in the recipes
Acorus gramineus L.	3
Cassia fistula L.	3
Cyperus rotundus L.	3
Dregea volubilis (L.f.) Benth. Ex Hook.f.	3
Eclipta prostrate L.	3
Myristica fragrans Houtt.	4
Nigella sativa L.	4
Plumbago indica L.	6
Piper nigrum L.	8
Pellacalyx parkinsonii Fisch. ST	3
Piper sarmentosum Roxb.	3
Plumbago zeylanica L.	4
Terminalia chebula Retz.	8
Tinospora crispa L.	9
Zingiber officinale Roscoe	9

Figure 7 *Acorus gramineus* L. (Family: Araceae) [online] Available:http:// www.vienduoclieu.org.vn [2010, September 1]

1.4.4.2 *Cassia fistula* L. (**Figure 8**)

This plant has Thai common name of "Khuun or ratchaphruek". The rip pods and seeds of *C. fistula* are used as a laxative. Other plant parts (root-bark, leaves and flowers) are also had purgative properties. The bark is reported to be used to treat skin problem, whereas the leaves are applied on fungal skin infections. In Panama *C. fistula* is used in folk medicine for the treatment of diabetes (Monif et al., 1992; Lemmens and Bunyapraphatsara, 1999). The fruit of *C. fistula* is rich in pectins and mucilages. The laxative properties are mainly due to the content of antraquinone derivatives, for examples, rhein, sennidins and sennosides. The sugar moiety in the glycosides increase water solubility of the molecule, and thus facilitates transport to the site of action (the colon). In the colon, bacteria hydrolyze the glycosides and dianthrones to anthraquinones, a reaction which is immediately followed by the local reduction of the antraquinones to their corresponding anthrones. The latter

compounds act directly on colon, to stimulate peristasis (Asselein et al., 1990). The methanol extract of *C. fistula* has been evaluated in *in vitro* models in protecting free radical-induced lipid peroxidation in model membranes (Sunil and Muller, 1998). Flavonoids in this plant have also been reported to inhibit tyrosinase due to their ability to chelate copper in the active site (Maity et al., 1998; Cuellar et al., 2001). The fruit tissue of this plant was found to be a rich source of potassium, calcium, iron and manganese (Barthakur et al., 1995) which may be competitive inhibitors of the tyrosinase enzyme. Proanthocyanidins including flavan-3-ol (epiafzelechin and epicatechin) units with an abnormal 2*S*-configuration together with the common flavan-3-ols and proanthocyanidins like catechin, epicatechin, procyanidin B-2 and epiafzelechin which are strong tyrosinase inhibitors have also been found in the pods of *C. fistula* (Kashiwada et al., 1990).

Figure 8 *Cassia fistula* L. (Family: Fabaceae) [online] Available:http://www.payer.de/ayurveda/caraka0101074.htm [2010, September 1]

1.4.4.3 *Cyperus rotundus* L. (**Figure 9**)

This plant has Thai common name of “Ya haeo mu”. *C. rotundus* is a traditional herbal medicine used widely as analgesic, sedative, antispasmodic, antimalarial, stomach disorders and to relieve diarrhoea (Zhu et al., 1997). The tuber part of *C. rotundus* is one of the oldest known medicinal plants used for the treatment of dysmenorrheal and menstrual irregularities (Bhattarai, 1993). The major chemical components of this herb are essential oils, flavonoids, terpenoids, mono-and sesquiterpenes (Ohira et al., 1998). *C. rotundus* has been reported to contain many phenolic compounds, such as gallic acid, *p*-coumaric acid and epicatechin (Proestos et al., 2005) which are potent antioxidants by ferric reducing antioxidant power and Trolox equivalent antioxidant capacity assays along with metal chelating properties (Amin and Razieh, 2007).

Figure 9 *Cyperus rotundus* L. (Family: Cyperaceae) [online] Available:http:// www.vienduoclieu.org.vn [2010, September 1]

1.4.4.4 *Dregea volubilis* (L.f.) Benth. Ex Hook.f. (**Figure 10**)

This plant has Thai common name of “Kra thung ma ba”. *D. volubilis* is extensively used to treat inflammation, piles, leucoderma, asthma, tumors, urinary discharge etc. (Kirtikar and Basu, 1935). Drevogenin D and kaempferol have been isolated from its leaves whereas dregeosides, hyperoside, drevogenin A and P as also drebbysogenin were isolated from its seeds, stem and roots, respectively (Yoshimura et al., 1983). Furthermore, Sahu et al., (2002) isolated three novel polyoxypregnane glycosides volubilioside A, B, C along with drevogenin D and P from the flowers. Additionally, Biswas et al., (2009) isolated an anti-inflammatory pentacyclic triterpenoid taraxerol from fruits of the plants.

Figure 10 *Dregea volubilis* (Family: Asclepiadaceae) [online] Available:http://www.jircas.affrc.go.jp/project/value_addition/Vegetables/048.html [2010, September 1]

1.4.4.5 *Eclipta prostrate* L. (**Figure 11**)

This plant has Thai common name of “Ka meng”. *E. prostrate* has been used as a traditional medicine to treat hyperlipidemia, atherosclerosis, hepatic disorders, spleen enlargement, and skin diseases (Anon, 1952).

Figure 11 *Eclipta prostrate* L. (Family: Asteraceae) [online] Available:http://http://www.rakbankerd.com/agriculture/page?id=1586ands=tblplant [2010, September 1]

1.4.4.6 *Myristica fragrans* Houtt. (**Figure 12**)

This plant has Thai common name of “Chan thet”. *M. fragrans* has been reported to have various biological activities, including antioxidant, anti-inflammatory, anticariogenic, and antihepatotoxic properties (Filleur et al., 2001; Sadhu et al., 2003). *M. fragrans* has been reported to contain 25–30% fixed oils and 5–15% volatile oils such as camphene, elemicin, eugenol, isoelemicin, isoeugenol, methoxyeugenol, pinene, sabinene, safrol, etc., and also chemical substances such as dihydroguaiaretic acid, elimicin, myristic acid, myristicin and lignan compounds

(Isogai et al., 1973; Kuo, 1989; Janssen et al., 1990). In skin, macelignan has been shown to have an inhibitory effect on melanin overproduction and accumulation in murine melanocytes suggesting that it may have benefits as a skin-whitening agent (Cho et al., 2008).

Figure 12 *Myristica fragrans* Houtt. (Family: Myristicaceae) [online] Available:http:// www.botanical.com/botanical/mgmh/n/nutmeg07.html [2010, September 1]

1.4.4.7 *Nigella sativa* L. (**Figure 13**)

This plant has Thai common name of “Thian daeng”. *N. sativa* has been used as a spice, food additive, preservative, as well as herbal remedy for various diseases and conditions such as asthma, diarrhea, headache, toothache, nasal congestion, and several types of cancer (Ali and Blunden, 2003; Salem, 2005). The oil extracted from seeds contains saturated and unsaturated fatty acids and 1.1–1.4%

volatile oil, major components of which are thymoquinone and nigellone (polythymoquinone) (El-Dakhakhny, 1963).

Figure 13 *Nigella sativa* L. (Family: Ranunculaceae) [online] Available:http:// www.impgc.com/plantinfo_A.php?id=746 [2010, September 1]

1.4.4.8 *Plumbago indica* L. (**Figure 14**)

This plant has Thai common name of "Chetta mun phloeng daeng". The dried root is credited with emmenagogue, stomachic and carminative activities and it is reported to be used in the treatment of heamorrhoids, as an abortifacient and as a means to purified the blood and stimulates digestion (Lemmens and Bunyapraphatsara, 1999). *P. indica* can be largely attributed to the presence of plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone) which is mainly extracted from the roots and is only found in *Plumbaginaceae* (Lemmens and Bunyapraphatsara, 1999).

Figure 14 *Plumbago indica* L. (Family: Plumbaginaceae) [online] Available:http:// http://www.flickr.com/photos/catalogthis/3708301021/ [2010, September 2]

1.4.4.9 *Piper nigrum* L. (**Figure 15**)

This plant has Thai common name of "Phrik thai". The pepper, the fruits of *P. nigrum*, is important as spice and flavoring agents, and has also been used in the treatment of cholera and dyspepsia, as well as a variety of gastric ailments and arthritic disorders (Jung and Shin, 1998). Terpenes, steroids, lignans, flavones, and alkaloids/alkamides have been identified as the primary constituents of the peppers (Parmar et al., 1997; Navickene et al., 2000).

1.4.4.10 *Pellacalyx parkinsonii* Fisch. ST (**Figure 16**)

This plant has Thai common name of "Kan phlu". Bud oils of *P. parkinsonii* have biological activities, such as antibacterial, antifungal, insecticidal and antioxidant properties, and are used traditionally as flavoring agent and antimicrobial material in food (Huang et al., 2002; Velluti et al., 2003). The volatile

oil of this plant (about 15%), which is composed of basically four substances: eugenol, β-caryophyllene, α-humulene and eugenyl acetate. The first is the main compound in clove oil, representing over 50% of the total extract composition (Guan et al., 2007).

Figure 15 *Piper nigrum* L. (Family: Piperaceae) [online] Available:http:// http://www.uni-graz.at/~katzer/engl/Pipe_nig.html [2010, September 2]

Figure 16 *Pellacalyx parkinsonii* Fisch. ST (Family: Rhizophoraceae) [online] Available:http:// http://veg888.wordpress.com/2009/12/15/recipe-for-illness-part-3-2/ [2010, September 2]

1.4.4.11 *Piper sarmentosum* Roxb. (**Figure 17**)

This plant has Thai common name of “Cha phlu”. *P. sarmentosum* has also been used as a carminative, expectorant and to relieve muscle pains and coughs (Apisariyakul, 1984). The plant has been shown to have antiplatelet aggregation (Han et al., 1992) and antibacterial effects (Silpasuwon, 1979). Six chemical constituents of *P. sarmentosum* leaves and fruits, extracted with petroleum ether, were hydrocinnamic acid, β-sitosterol, pellitorine, pyrrole amide, sarmentine and sarmentosine (Niamsa and Chantrapromma, 1983; Likhitwitayawuid et al., 1987; Strunz and Finlay, 1995).

Figure 17 *Piper sarmentosum* Roxb. (Family: Piperaceae) [online] Available:http:// prathom.swu.ac.th/panmai/show_img.asp?ID=7-10110-002-285.2 [2010, September 2]

1.4.4.12 *Plumbago zeylanica* L. (**Figure 18**)

This plant has Thai common name of “Chetta mun phloeng khao”. *P. zeylanica* has been described for significant anticancer, antitumor, anti-inflammatory and antimicrobial activities. The plant is also effective against rheumatic pain, sprains,

scabies, skin diseases and wounds (Nguyen et al., 2004; Rahman and Anwar, 2007). The roots of the plant and its constituents are credited with potential therapeutic properties including antiatherogenic, cardiotonic, hepatoprotective, neuroprotective and central nervous system stimulating properties (Bopaiah and Pradhan, 2001; Tilak et al., 2004). Naphthoquinones, binaphthoquinones, flavonoids, coumarins, alkaloids, glycosides, tannin, saponins and steroids have been isolated from *P. zeylanica* (Gunaherath et al., 1983, Dinda and Saha, 1989; Lin et al., 2003).

Figure 18 *Plumbago zeylanica* L. (Family: Plumbaginaceae) [online] Available:http:// www.mozambiqueflora.com/speciesdata/image-display.php?species_id=143690&image_id=1 [2010, September 2]

1.4.4.13 *Terminalia chebula* Retz. galls (**Figure 19**)

This plant has Thai common name of “Kot phung pla”. Its medical applications include astringent, purgative, supplements for anti-aging and impartment of longevity as well as boosting of the immune system (The Pharmacopoeia Commission of PRC, 1997; Zhu, 1998; Lemmens and Bunyapraphatsara, 2003). Moreover, *T. chebula* fruits have been shown to have antioxidant (Chen et al., 2003),

antimicrobial (Burapadaja and Bunchoo, 1995), and anticancer activities (Lee et al., 1995; Saleem et al., 2002). Fourteen components of hydrolyzable tannins including gallic acid, chebulic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulagic acid, chebulinic acid, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, 1,6-di-*O*-galloyl-D-glucose, casuarinin, 3,4,6-tri-*O*-galloyl-D-glucose, and terchebulin have been found in *T. chebula* fruits (Juang et al., 2004).

Figure 19 *Terminalia chebula* Retz. (Family: Combretaceae)

1.4.4.14 *Tinospora crispa* L. (**Figure 20**)

This plant has Thai common name of "Boraphet". Stems and leaves of *T. crispa* are used in anti-inflammatory (antirheumatic), antimalarial and antibacterial properties and treatment of skin complaints such as antiseptic, antiparasitic and for wound and itches. A number of chemical constituents have already been isolated from different parts of *T. crispa*, for examples, diterpenes, alkaloids and flavonoids. Most extensively investigated are a series of furano-diterpene (glycoside) compounds: tinotufolin (leaves), borapetoside (stem) and their bitter tinocrisposide (stem). The alkaloids isolated from *T. crispa* are palmatine (stem), berberine (stem and root) and

aporphine. Of these isolated alkaloids, berberine and its salts are known to have spasmolytic, antibacterial and antifungal activity (Acevedo et al., 1970; Pachaly and Adnan, 1989).

Figure 20 *Tinospora crispa* L. (Family: Menispermaceae) [online] Available:http:// www.tratcc.ac.th/wwwstd/14samoonpai/page_ton_03.html [2010, September 2]

1.4.4.15 *Zingiber officinale* Roscoe (**Figure 21**)

This plant has Thai common name of "Khing". Stems and leaves of *Z. officinale* are used in anti-inflammatory (antirheumatic), antimalarial and antibacterial properties and treatment of skin complaints such as antiseptic, antiparasitic and for wound and itches. *Bioactive compounds:* Its essential oil, which is a mixture of monoterpenic and sesquiterpenic compounds, contains the volatile compounds responsible for the characteristic ginger flavor and α-zingiberene is its major component. The oleoresin contains the volatile substances (volatile oil) and substances responsible for the pungent flavor of fresh ginger; these substances, which are phenolic ketones, are known as 4-, 6-, 8-, 10-, and 12-gingerol (He et al., 1998). 6-

Gingerol has been reported to exhibit antioxidative activity against linoleic acid autoxidation and peroxidation of phospholipid liposomes and to scavenge trichloromethylperoxyl- and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (Aeschbach et al., 1994; Sekiwa et al., 2000).

Figure 21 *Zingiber officinale* Roscoe (Family: Zingiberaceae) [online] Available:http://www.rakbankerd.com/agriculture/open.php?id=1082&s=tblplant [2010, September 2]

1.4.5 Problems of bioactive compounds from plants in pharmaceutical and cosmetic applications

1.4.5.1 Chemical stability

Most bioactive compounds from plants can be readily oxidized and degraded by oxygen, sun light and heat, thereby changing the compounds to have different properties. For examples, carotenoids, β-carotene and lycopene may have antioxidant properties, while their degraded products can be toxic in cell culture (Yeh and Hu, 2001). Flavonoids and other plant polyphenolics are also easily oxidizable

(Long et al., 2000). Drying of the plant material can cause the loss of thermolabile constituents and further loss can occur on storage, especially if the storage conditions are moist or too hot.

1.4.5.2 Formulation incompatibility

The plant extract may contain multiple different bioactive compounds which can interact with each other in unexpected ways, or can influence the effects of drugs taken concurrently such as garlic extracts affect the metabolism of paracetamol and warfarin, and kava can interact with drugs used in the treatment of Parkinson's disease (Izzo and Ernst, 1999). Moreover, bioactive compounds from plants may be incompatible with the formulation compositions. Polyphenolics and fatty acid, which acidic property (pH 3–4), can reduce the viscosity of cream or gel formulations. The color of bioactive compounds from plants contained in the formulation may give undesirable color.

1.4.5.3 Skin irritation

Some bioactive compounds from plants such as salicylate, thymol and phenolics can be toxic to skin and cause *in vivo* skin irritation if they are used in substantial amounts or for too long time. A number of phenolics appear in many plants. They are more complicated chemical structures which many hydroxyl groups resulting acidic property. Moreover, some phenolics occur in two forms, which are *trans*-anethole and *cis*-anethole. The latter is more toxic than the former (Frosch et al., 1992; Roberts et al., 1997).

1.4.5.4 Transdermal absorption

Many bioactive compounds especially polyphenolic compounds exhibited low skin permeation owing to their large molecular structures and

hydrophilic property. Hence, phenolic compounds in plants such as *T. chebula* galls have low skin permeation and will be obstacles for an efficient topical use in cosmetic and pharmaceutical applications.

1.4.6 Phytochemicals

1.4.6.1 Alkaloids

Alkaloids are nitrogen-containing compounds widely distributed in different plants. Alkaloids are normally grouped on the basic of their ring system. Several common ring systems, including indolizidine- and quinolizidine-based systems and quinoline-, quinazoline-, and acridone-based systems were recently reviewed (Michael, 2004). Alkaloids in the form of plant extracts have been used for poisons, narcotics, stimulants and medicines for several thousand years. The common examples include caffeine, quinine, nicotine, cocaine, morphine and strychnine **(Figure 22**). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (potassiomercuric iodide solution) and gives cream colored precipitate while Dragendorff's reagent (solution of potassium bismuth iodide) gives an orange colored precipitate with alkaloids.

1.4.6.2 Flavonoids

Flavonoids have two benzene rings separated by a propane unit. They are generally water-soluble compounds. The more conjugated compounds are often brightly colored. They are found in plants as their glycosides, which have complicated structure. The different classes within the group are distinguished by the additional oxygen-containing heterocyclic rings and hydroxyl groups. These include the chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavones (**Figure 23**) (Williams and Grayer, 2004).

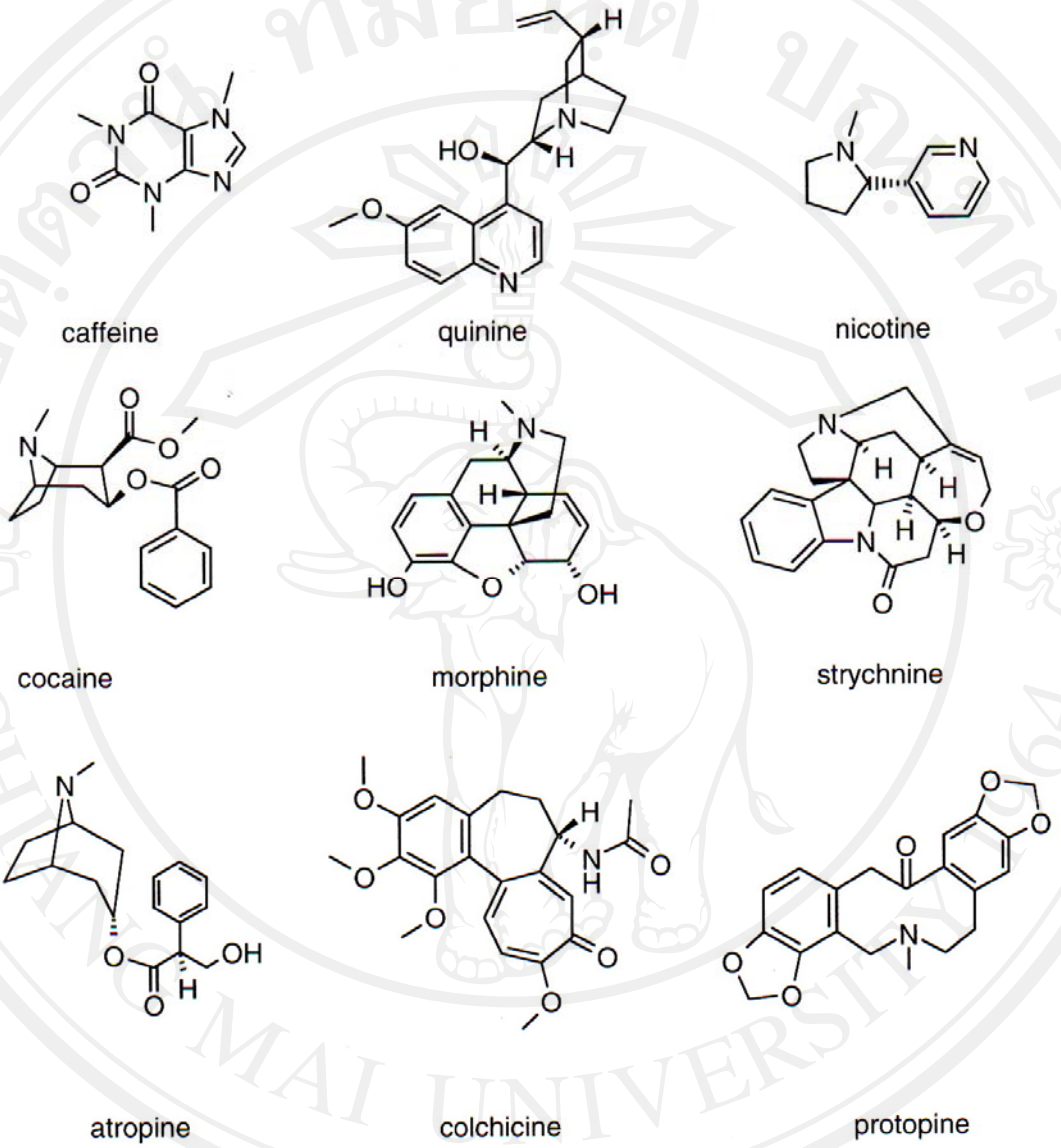


Figure 22 Chemical structures of some common alkaloids (Michael, 2004)

Flavonoids can be detected by using the alcoholic solution with few fragments of magnesium ribbon and concentrated hydrochloric acid. The magenta color will produce after few minutes (Shinoda test) (Harborne, 2005).

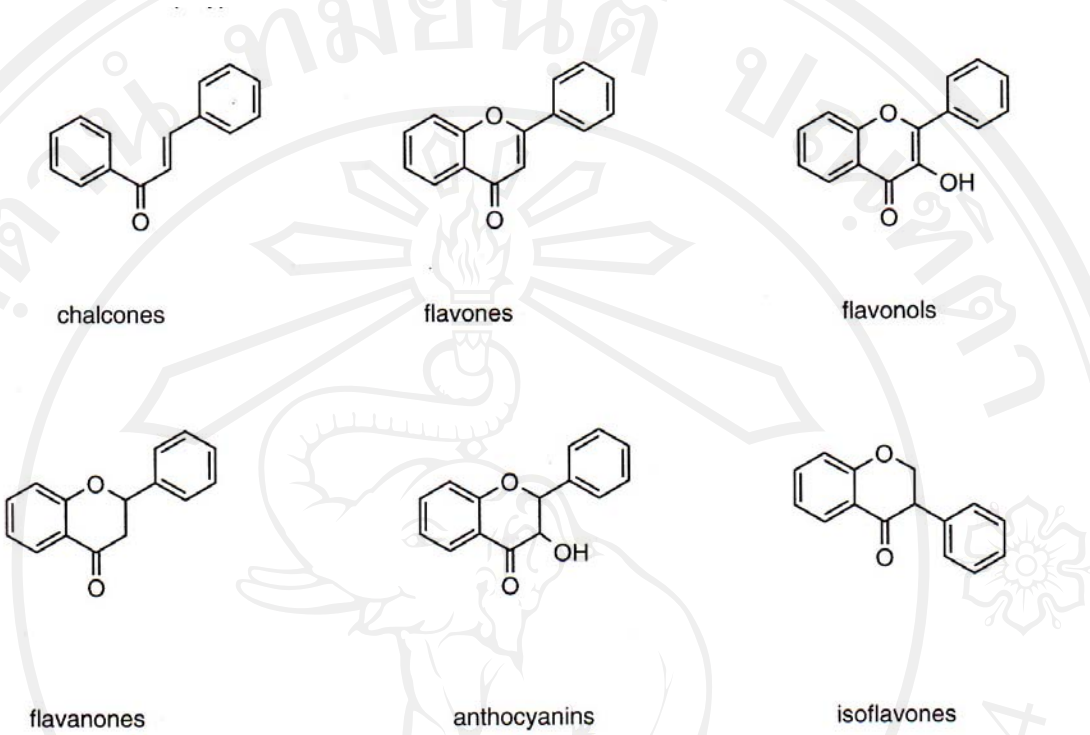


Figure 23 Chemical structures of flavoniod classes (Williams and Grayer, 2004)

1.4.6.3 Saponins

Saponins are high-molecular-weight triterpene glycosides, containing a sugar group attached to either a sterol or triterpene. Saponins are composed of two parts which are glycone (sugar) and aglycon or genin (triterpene). Typically, they have detergent properties, readily form foams in water, have a bitter taste, and toxic to fish. Many plants that contain saponins were historically used as soaps. These include Soaproot (*Chlorogalum pomeridianum*), Soapbark (*Quillaja saponaria*) and Soapnut (*Sapindus mukurossi*) (Hostettman and Marston, 1995). The aglycons may be triterpene, steroid or steroid alkaloid class. Representative saponins were presented in **Figure 24**.

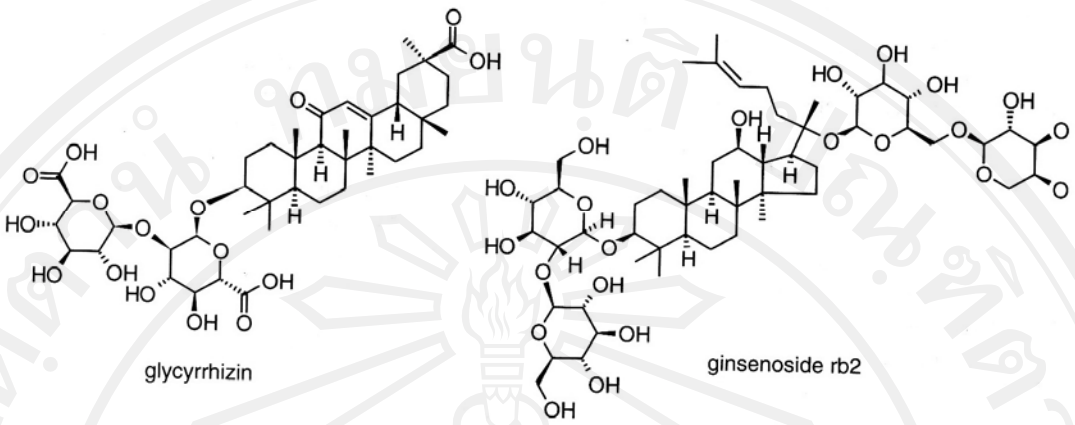


Figure 24 Chemical structures of saponins (Hostettman and Marston, 1995)

1.4.6.4 Tannins

Tannins are water-soluble oligomers, rich in phenolic groups, capable of binding or precipitating water-soluble protein. The tannins, common to vascular plants, exist primarily within woody tissues but can also be found in leaves, flowers or seeds. Plant tissues that are high in tannin contents have a highly bitter taste. Tannin may be divided into two groups which are either condensed tannins or hydrolysable tannins (**Figure 25**). Hydrolyzable tannins are ester of a sugar with one or more trihydroxy benzene carboxylic acids (gallic acid). These tannins give insoluble precipitates with albumin, starch or gelatin. This reaction with proteins is used industrially to convert animal skins into leather. Tannins produce different colors with ferric chloride (either blue, blue black, or green to greenish black) according to the type of tannin (Hagerman and Butler, 1989).

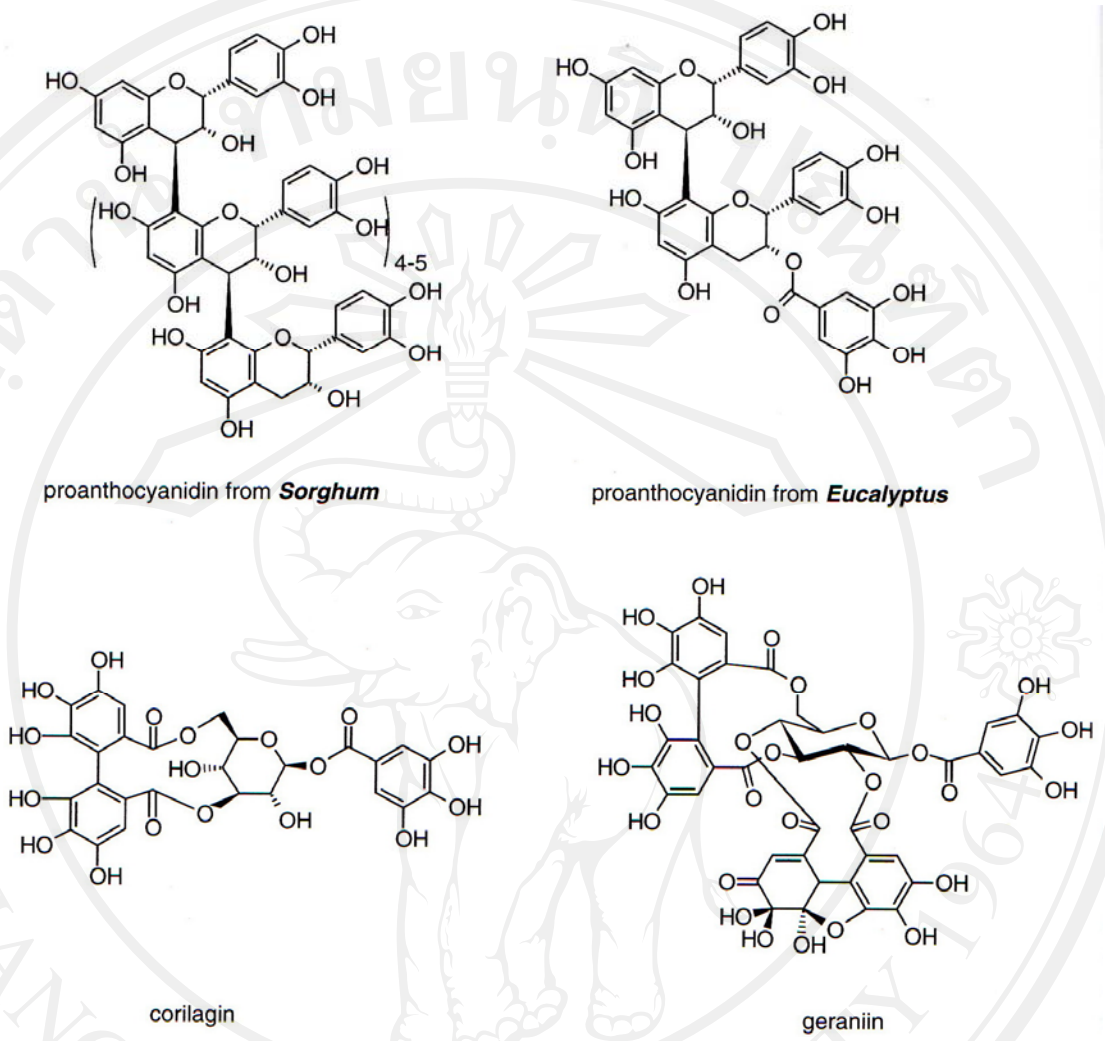


Figure 25 Chemical structures of condensed (proanthocyanins) and hydrolysable (corilagin and geraniin) tannins (Hagerman and Butler, 1989)

1.4.6.5 Reducing sugars

A reducing sugar is any sugar that, in a solution, has an aldehyde or a ketone group (**Figure 26**). This allows the sugar to act as a reducing agent, for example in the Maillard reaction and Benedict's reaction. A sugar is only a reducing sugar if it has an open chain with an aldehyde or a ketone group. Many sugars occur in a chain as well as in a ring structure. In solution, it is possible to have equilibrium

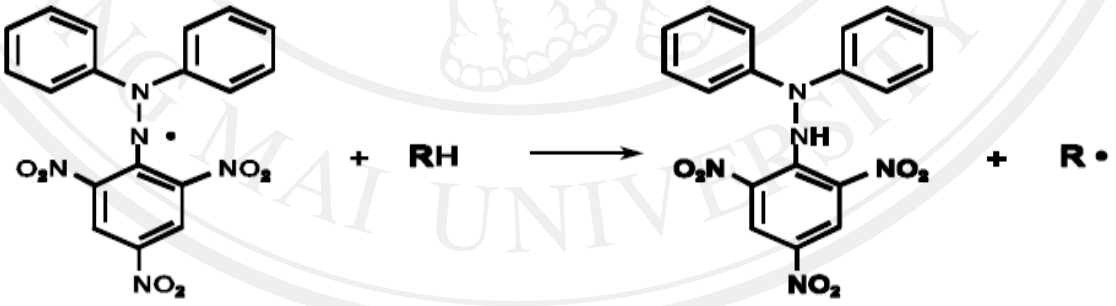
between these two forms. The aldehyde can be oxidized via a redox reaction. The chemical that causes this oxidation becomes reduced. Thus, a reducing sugar is one that reduces certain chemicals. Even though a ketone cannot be oxidized directly, a keto sugar may be converted to an aldehyde via a series of tautomeric shifts to migrate the carbonyl to the end of the chain. Reducing monosaccharides include glucose, fructose and galactose. Benedict's reagent and Fehling's solution are used to test for the presence of a reducing sugar. The reducing sugar reduces copper (II) ions in these test solutions to copper (I), which then forms a brick red copper (I) oxide precipitate. 3,5-Dinitrosalicylic acid is another test reagent that allows quantitative spectrophotometric measurement of the amount of the reducing sugar. Sugars having acetal or ketal linkages are not reducing sugars, as they do not have free aldehyde chains. Therefore, they do not react with any of the reducing-sugar test solutions. However, a non-reducing sugar can be hydrolysed using dilute hydrochloric acid. After hydrolysis and neutralization of the acid, the product may be a reducing sugar that gives normal reactions with the test solutions.

Figure 26 Chemical structures of reducing forms of glucose (left) and fructose (right)

1.4.7 *In vitro* biological assays to evaluate anti-aging activity

1.4.7.1 DPPH free radical scavenging activity assay

The free radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydracyl), a stable free radical, has been widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) or hydrogen donating activities of various compounds since it is a simple, rapid and sensitive method (Suja et al., 2005; Letelier et al., 2008). DPPH, a radical generating substance, has a deep violet color due to its unpaired electron. Free radical scavenging ability can be followed by the loss of the absorbance at 515 nm as the pale yellow non-radical form is produced. After DPPH solution react with the samples, the absorbance of the resulting solutions are measured and compared with the absorbance of DPPH in the absence of sample solution. Lower absorbance represents higher activity. The reaction of the DPPH radical in the presence of the antioxidant compound during the DPPH assay is shown in **Figure 27**.



Deep violet color at 515 nm Pale yellow (non-radical form)

Figure 27 Reaction of the DPPH radical in the presence of the antioxidant during the DPPH assay (Prakash, 2001)

1.4.7.2 Ferrous metal chelating activity assay

The ferrous ion (Fe^{2+}) is one of the most powerful pro-oxidants in lipid oxidation. Thus, the effective Fe^{2+} chelators may afford protection against oxidative damage in lipid peroxidation (Liyana-Pathirana and Shahidi, 2007). Fe^{2+}-ferrozine complex method is commonly used for the evaluation of chelating properties of the compounds. In the presence of chelating agents, the complex formation is disrupted resulting in a decrease in the red color of the complex which is followed by spectrophotometrically at 562 nm. Lower absorbance indicates higher metal chelating activity (Gulcin, 2006).

1.4.7.3 Tyrosinase inhibition assay

The human epidermis is composed of three important cell types including melanocytes, keratinocytes and Langerhans cells. Melanocytes are located in the basal layer in the epidermis. The melanocytes produce melanin to protect the skin from UV radiation. The process by which melanin is formed is called melanogenesis. Two basic types of melanin are eumelanin, which is a black pigment and pheomelanin which is a yellow to red pigment. The varieties of human skin color depend on the amount of eumelanin and pheomelanin. The melanogenesis pathway has been elucidated by Sung-Yum et al., 2003 (**Figure 28**). The process starts from the hydroxylation of L-tyrosine (amino acid) to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to L-DOPA quinone. These steps are under enzymatic control while the remaining steps occur spontaneously. The tyrosinase, copper-containing protein, is a key enzyme for melanogenesis. Tyrosinase existing in the skin can catalyze the oxidation of L-tyrosine to L-DOPA and subsequently to L-DOPA quinone which will mediately change to melanin, a brown to black color

pigment. Therefore, any compounds which can inhibit this enzyme, can inhibit the formation of melanin.

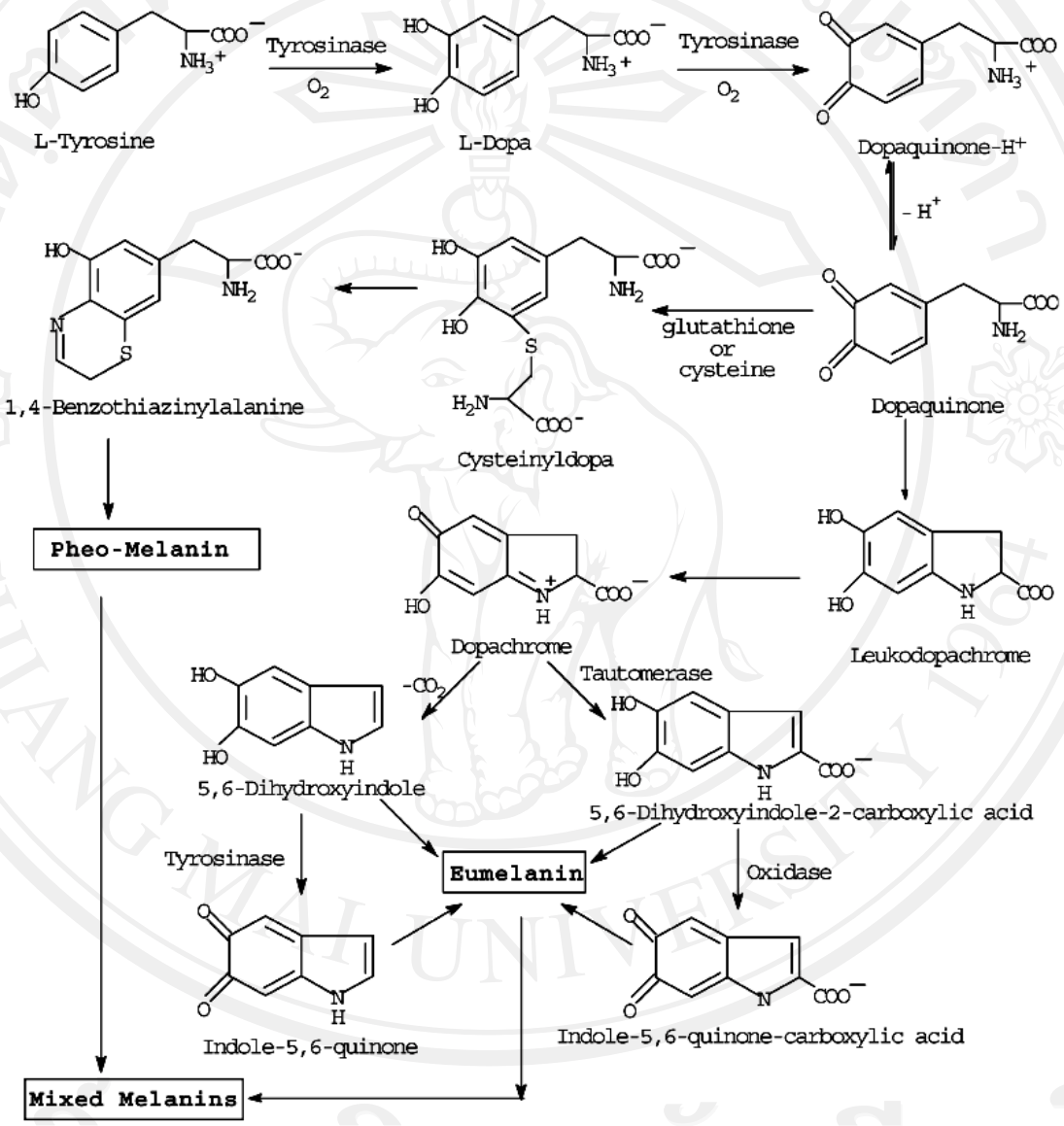


Figure 28 Melanogenesis pathway (Sung-Yum et al., 2003)

1.4.7.4 Nitric oxide inhibition assay

Nitric oxide (NO) is gaseous free radical that plays important roles in living organisms. In animals, NO originates from the oxidation of L-arginine to L-citruline in a complex process catalyzed by nitric oxide synthase (NOS) enzymes (**Figure 29**). Nicotinamide adenine dinucleotide (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (H_4B) and calmodulin function as cofactors for NOS activity (Bredt and Snyder, 1990).

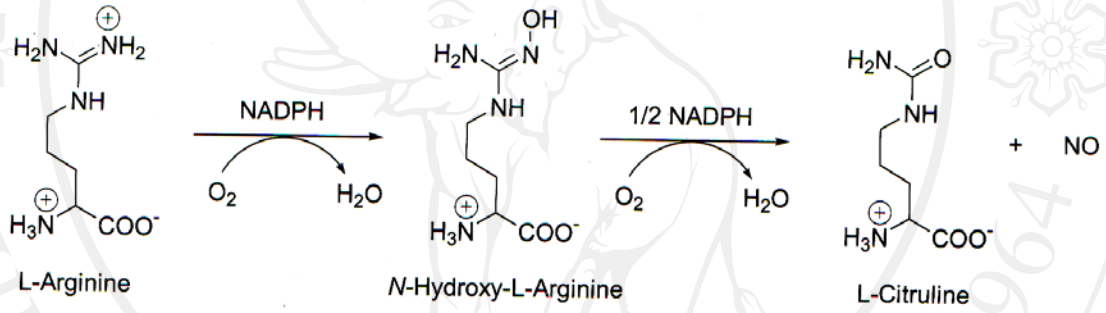


Figure 29 Nitric oxide (NO) biosynthesis catalyzed by nitric oxide synthease (NOS) enzyme (Bredt and Snyder, 1990)

Enzymes nNOS, iNOS and eNOS are the three main isoforms, named according to the tissues where they were first identified which are brain; activated macrophages and vascular endothelium, respectively. The nNOS and eNOS isoforms catalyze NO production in moderate amounts for normal signaling purposes while iNOS acts to produce large amounts of this free radical, leading to cytotoxic effect. Uncontrolled overproduction of NO can contribute to septic shock, cancer, inflammatory process and neurodegenerative diseases such as Parkinson's and Alzheimer's diseases

(Chabrier et al., 1999; Ying and Hofseth, 2007). Many plant natural products have been shown either to inhibit mammalian NOS activities or to decrease the transcription levels of NOS genes. Hydrolysable tannin extracts from *Melastoma dodecandrum* (**107**–**109**, and **111**) (**Figure 30**) together with the compound **110** caused the reduction in the NO levels in LPS-activated RAW 264.7 macrophage cells (Ishii et al., 1999). Compound **110** showed less inhibitory than compounds **107**–**109** and **111**. This suggested that the conjugation of the functional galloyl or hexahydroxydiphenol groups was necessary for the inhibitory activity.

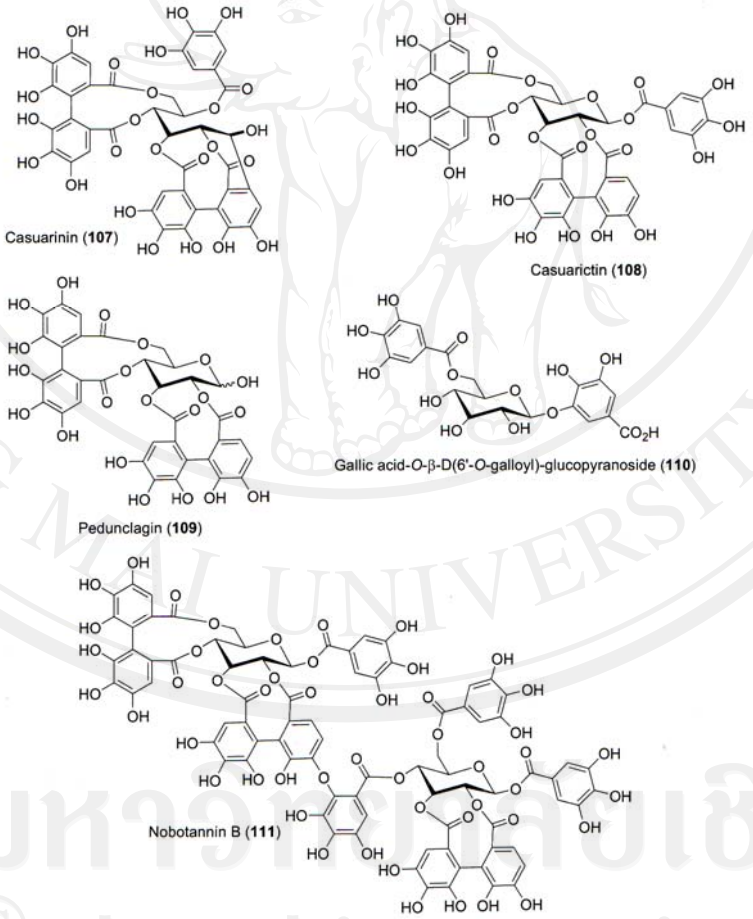


Figure 30 Structures of natural products isolated from *Melastoma dodecandrum* that present inhibitory activity on NO production in activated macrophages (Ishii et al., 1999)

1.4.7.5 Cell proliferation activity

In vitro cell proliferation assay in cancer cell has been recognized as a primary tool for the screening of anticancer agents. Mosmann (1983) established the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and subsequently, modified tetrazolium salts like XTT and WST-1 have become available. Using these assays, a large number of tests can be carried out in a rapid, reproducible and sensitive fashion. The development of the sulforhodamine B (SRB) protein binding assay for the *in vitro* measurement of cellular protein content of the adherent and suspension cultures for the evaluation of cytotoxicity and cell proliferation in microplate was established by Skehan et al. (1990). Subsequently, it was adopted for routine use in the National Cancer Institute (NCI, USA) *in vitro* antitumor screening.

A. MTT assay

Colorimetric MTT (tetrazolium) assay which was described by Mosmann (1983) has been developed for a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cell. This method can therefore be used to measure cytotoxicity, proliferation or activation. The main advantages of the colorimetric assay are its rapidity, precision and lack of any radioisotope. The principle of MTT assay is that the yellow tetrazolium MTT is reduced by metabolically active cells in the part by the action of dehydrogenase enzymes in mitochondria. The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells. The resulting intracellular dark blue formazan crystal can be solubilized and quantified by a multiwell spectrophotometer

(microplate reader). The structure of MTT and their corresponding reaction products are shown in **Figure 31**.

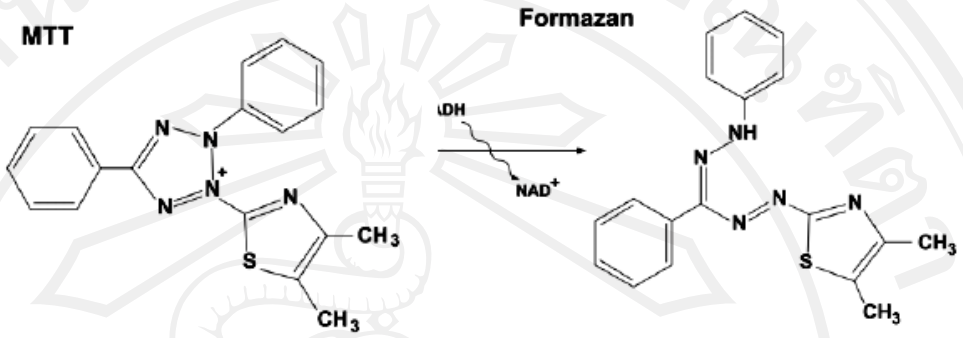


Figure 31 Structure of MTT and their corresponding reaction products (Mosmann, 1983)

B. SRB assay

SRB is a bright pink aminoxanthene dye. It posseses 2 charged SO_3 groups which are capable of electrostatically binding to positive counter ions. Under mildly acidic conditions, SRB binds to the positive fixed charges of biological molecules. In TCA fixed cells, these binding sites are primarily the amino groups of the proteins. The SRB binds to the basic amino acids of cellular macromolecules and the colorimetric evaluation provides an estimation of total protein mass which is related to cell growth or viability in the treated and untreated cells. SRB behaves like a bromphenol blue, naphthol yellow S, and Coomassie Blue, which are also used widely as protein stains. With TCA fixed cultures, SRB gives a higher OD and better signal to noise ratio at low cell density than do these other dyes. The method has replaced the tetrazolium based assays by exhibiting a number of advantages including

better linearity, higher sensitivity, a stable end point that does not require time-sensitive measurement and lower cost and toxicity (Papazisis et al., 1997).

1.4.7.6 Gelatinolytic activity on MMP-2 inhibition (Zymography)

A. Types of collagen and MMPs

Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. More than 90% of the extracellular protein in the tendon and bone and more than 50% in the skin consist of collagen (Piez, 1985). Different collagen types are necessary to confer distinct biological features to various types of connective tissues in the body. Collagen comprises a family of genetically distinct molecules which have a unique triple-helix configuration of three polypeptide subunits known as α-chains in common. Currently, at least 13 types have been isolated which vary in the length of the helix and the nature and size of the non-helical portions (**Table 6**) (Kucharz, 1992).

MMPs are a family of 28 proteolytic endopeptidases (**Table 7**) containing a Zn (II) ion held in the active site by three histidines (Sternlicht and Werb, 2001). This family is called matrix metalloproteinases because of their ability to degrade the extracellular matrix (ECM). Their dependence on a metal ion (Zn II) for their activity and their sequence and domain similarities distinguish them from other closely related metalloprotinases. MMPs are primarily involved in tissue remodeling, but are also implicated in cell-signaling by releasing of growth factors, aging, embryogenesis, angiogenesis, endometrial maintenance and bone remodeling (Schulz, 2007). As well as cancer, MMPs are also implicated in a number of diseases like rheumatoid arthritis, periodontal disease and intestinal inflammation (Jacobsen et al, 2007; Schulz, 2007).

Table 6 Body distribution of collagen types (Kucharz, 1992)

Collagen type	Tissue distribution
I	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dentin, dermis, tendon
II	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except in bone, tendon, cornea)
IV	Basement membranes
V	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	Skin, placenta, lung, cartilage, cornea
VIII	Produced by endothelial cells, Descemet's membrane
IX	Cartilage
X	Hypertrophic and mineralizing cartilage
XI	Cartilage, intervertebral disc, vitreous humour
XII	Chicken embryo tendon, bovine periodontal ligament
XIII	Cetal skin, bone, intestinal mucosa

Table 7 Types of MMPs (Snoek-van Beurden and Von den Hoff, 2005)

Subgroup	MMP	Name	Substrate
1. Collagenases	MMP-1	Collagenase-1	Col I, II, III, VII, VIII, X, gelatin
	MMP-8	Collagenase-2	Col I, II, III, VII, VIII, X, aggrecan, gelatin
	MMP-13	Collagenase-3	Col I, II, III, IV, IX, X, XIV, gelatin
2. Gelatinases	MMP-2	Gelatinase A	Gelatin, Col I, II, III, IV, VII, X
	MMP-9	Gelatinase B	Gelatin, Col IV, V
3. Stromelysins	MMP-3	Stromelysin-1	Col II, IV, IX, X, XI, gelatin
	MMP-10	Stromelysin-2	Col IV, laminin, fibronectin, elastin
	MMP-11	Stromelysin-3	Col IV, fibronectin, laminin, aggrecan
4. Matrilysins	MMP-7	Matrilysin-1	Fibronectin, laminin, Col IV, gelatin
	MMP-26	Matrilysin-2	Fibrinogen, fibronectin, gelatin
5. MT-MMP	MMP-14	MT1-MMP	Gelatin, fibronectin, laminin
	MMP-15	MT2-MMP	Gelatin, fibronectin, laminin
	MMP-16	MT3-MMP	Gelatin, fibronectin, laminin
	MMP-17	MT4-MMP	Fibrinogen, fibrin
	MMP-24	MT5-MMP	Gelatin, fibronectin, laminin
	MMP-25	MT6-MMP	Gelatin
6. Others	MMP-12	Macrophage metalloelastase	Elastin, fibronectin, Col IV
	MMP-19		Aggrecan, elastin, fibrillin, Col IV, gelatin
	MMP-20	Enamelysin	Aggrecan
	MMP-21	XMMP	Aggrecan
	MMP-23		Gelatin, casein, fibronectin
	MMP-27	CMMP	Unknown
	MMP-28	Epilysin	Unknown

B. Collagen and MMPs in aging

Changes in the production and formation of collagen and elastic fibers are common characteristics of an aging dermis (El-Domyati et al., 2002). The relative proportion of the types of collagen in skin also changed with age. Young skin is composed of approximately 80% of type I collagen and about 15% of collagen type III. In an aged skin, collagen fibers became thicker and there was a loss of collagen type I, which altered the ratio of the collagen types (Oikarinen, 1990). Moreover, aged fibroblasts synthesized lower levels of collagen, both *in vitro* and *in vivo*, compared to young adult fibroblasts (Varani et al., 2006). Fisher et al. (2009) reported that dermal fibroblasts express higher levels of MMP-1 in aged human skin *in vivo*, when compared with fibroblasts from young skin. In addition, the annual loss of total collagen was approximately 1% from 50 years of age. Senescent fibroblasts exhibit phenotypic changes, becoming wide and flat with irregular shapes and lobulated nuclei (Kletsas, 2003). In an aged skin, there is an accumulation of elastosis and further degradation of the extracellular matrix due to the action of MMPs, which decreased collagen levels (Yin et al., 2000). MMPs (collagenase, gelatinase and stromelysin) can degrade collagen fibers, although collagen is relatively stable. Interstitial collagenase (MMP-1) produced by interstitial fibroblasts and inflammatory cells (macrophages and leukocytes) can degrade collagen types I and III. MMP-1 introduces a single break in each molecule of the triple α-chain, thus denaturing the collagen molecule. Metalloproteinases-9 (MMP-9) and metalloproteinases-2 (MMP-2) can degrade collagen IV (Gross and Lapiere, 1962). After the initial proteolytic cleavage, collagen can be degraded by other proteolytic enzymes (Horwitz et al., 1977; McCroskery et al., 1975). In general, this process is similar for all types of

collagenase. However, differences exist in the susceptibilities of different types of collagen to various collagenases. For example, MMP-1 cleaves type I collagen more rapidly than it cleaves type III collagen (Horwitz et al., 1977).

C. Zymography

Zymography is an electrophoretic technique based on SDS-PAGE that includes a substrate co-polymerized with the polyacrylamide gel, for the detection of enzyme activity (Lantz and Ciborowski, 1994). Samples are prepared in the standard SDS-PAGE treatment buffer but without boiling, and without a reducing agent. Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100, followed by incubation in an appropriate digestion buffer, for an optimized length of time at 37°C. The zymogram is subsequently stained (commonly with Amido Black or Coomassie Brilliant Blue), and the areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme (Snoek-van Beurden and Von den Hoff, 2005). Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9 (**Figure 32**). It is extremely sensitive because levels of 10 pg of MMP-2 can already be detected (Kleiner and Stetler-Stevenson, 1994). It should be considered, however, that other MMPs, such as MMP-1, MMP-8, and MMP-13 can also lyse the substrate. This signal will probably be very weak because gelatin is not their preferential substrate. For MMPs that do not show any activity on gelatin, modifications of the technique have been made for an improved detection. This is mainly done by incorporating a more suitable substrate into the gel, such as casein or collagen, or by enhancing the signal by adding heparin to the samples (Gogly et al., 1998; Yu and Woessner, 2001).

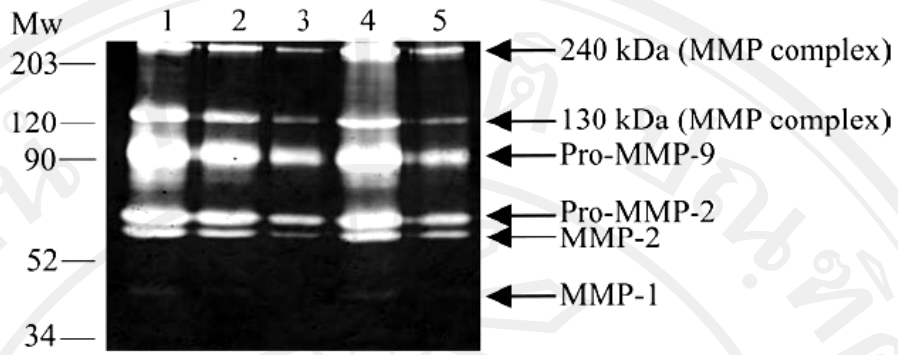


Figure 32 Gelatin zymography (Snoek-van Beurden and Von den Hoff, 2005)

D. Hemocytometer counting cell method

A variety of the methods has been used to measure the viability or proliferation of cells *in vitro*. Hemocytometer counting cell method is inexpensive and requires only a small fraction of total cells from a cell population. Therefore, this method is generally used to determine the cell concentration (cell number/ ml) in batch cell cultures. The hemocytometer consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted, so that the area bounded by the lines and the depth of the chamber are known. It is therefore possible to count the number of cells in a specific volume of fluid, thereby being able to calculate the concentration of the cells in the fluid overall.

1.4.8 *In vivo* biological assay

1.4.8.1 Animal testing for pharmaceutical activities

A. Rat ear edema test

Numerous rodent ear assays have been developed as models of inflammatory skin conditions. In general, these models are directed to mimic and

investigate the pathological components of dermatological diseases and discover new anti-inflammatory drugs. The majority of the animal models used in cutaneous inflammation research are based on the induction of acute or subacute cutaneous inflammatory responses by applying chemical agents such as croton oil, ethyl phenylpropiolate (EPP) and arachidonic acid. In these models, a vascular type of acute inflammation is induced (Young et al., 1984; Carlson et al., 1985; Bouclier et al., 1990). Allergic models in which an acute immune-type inflammation is provoked by sensitizing and the subsequent challenge of the animals with sensitizing agents such as oxazolone and 1-chloro-2,4-dinitrobenzene (DNCB) (Tarayre et al., 1984; Bouclier et al., 1990). All of these models can be used to evaluate anti-inflammatory activity of agents after topical or systemic administration. Erythema and particularly edema is generally used as an end point of inflammation evaluation. After topical application, materials are usually applied simultaneously at the same area, shortly before or after the application of the proinflammatory agent, easily assessed by ear thickness and ear biopsy weight measurements (De Young et al., 1989; Meurer et al., 1988).

B. Rabbit skin testing for irritation determination

In cosmetic industry, evaluation of irritancy potential to human skin of any chemicals or formulations is necessary. This must be done by the means of *in vivo* and *in vitro* tests to determine the risk of irritation due to the contact between these compounds and human skin. The most commonly used test is the rabbit skin irritation test described in the OECD test guideline 404 and in the European Chemicals Bureau Annex V part B.4 (http://ecb.jrc.it/testing-methods/) which was initially described by Draize et al. (1944). In this animal test, the test substances, either raw materials or finished formulated products, are applied on the rabbit's shaved skin. A score of skin

reactions is based on the physiological observations on the animals. After the tested products are applied on the rabbit skin, the rabbits are examined for the presence of erythema and oedema according to the Draize dermal irritation scoring system (0: no erythema or no edema; 1: barely perceptible erythema or edema; 2: well defined erythema or slight edema; 3: moderate to severe erythema or moderate edema; 4: severe erythema or edema) at the grading intervals of 1, 24, 48 and 72 h. The skin irritation potential of the chemicals is often summarized as the 'primary irritation index' (PII) calculated from the erythema and edema grades according to the following formula: PII = [(∑ *erythema grade at 24/48/72 h* + ∑ *edema grade at 24/48/72 h)/ 3* × *number of animals*]. The irritation degree was categorized based on the PII values as negligible (PII = 0–0.4), or slight (PII = 0.5–1.9), moderate (PII = 2–4.9) or severe (PII = 5–8) irritation.

1.4.8.2 Human volunteers for performance test

A. Skin elasticity measurement

Decreased skin elasticity is considered as a factor that promotes wrinkle formation which is the aged appearance. A Cutometer is used to measure the elastic properties of the skin. The measuring principle is based on the suction process. Low pressure is created in the device and the skin is drawn into the aperture of the probe. Inside the probe, the penetration depth is determined by a non-contact optical measuring system. This optical measuring system consists of a light source and a light receptor, as well as two prisms facing each other, which project the light from the transmitter to the receptor. The light intensity varies due to the penetration depth of the skin. The resistance of the skin to be sucked up by the low pressure (firmness) and its ability to return into its original position (elasticity) are displayed as the curves at

the end of each measurement (**Figure 33**). From these curves, interesting measurement parameters can be calculated. Multiple parameters: i.e., immediate distention (Ue), final distention (Uf), immediate retraction (Ur), delayed distention (Uv), the ability to return to the original position after deformation (Ur/Uf) and ratio between viscosity and elasticity (Uv/Ue) are frequently used to evaluate skin elasticity. During aging, skin elastic recovery (Ur/Uf) has been reported to decrease whereas, skin elastic extension (Uv/Ue) has been shown to increase progressively (Escoffier et al., 1989; Takema et al., 1994; Fujimura et al., 2007). These skin mechanical properties are related to the decrease in interstitial fluid viscosity as a result from low amount of glycosaminoglycans and soluble collagen as well as the decrease in elastic properties of collagen and elastin fibres due to the damage, disintegration, fragmentation, or changes of the protein fiber structure (Dobrev 1998; Dobrev 2002). Thus, an increase in Ur/Uf value (skin elastic recovery) and the reduction in Uv/Ue value (skin elastic extension) will indicate the improvement of skin elastic properties.

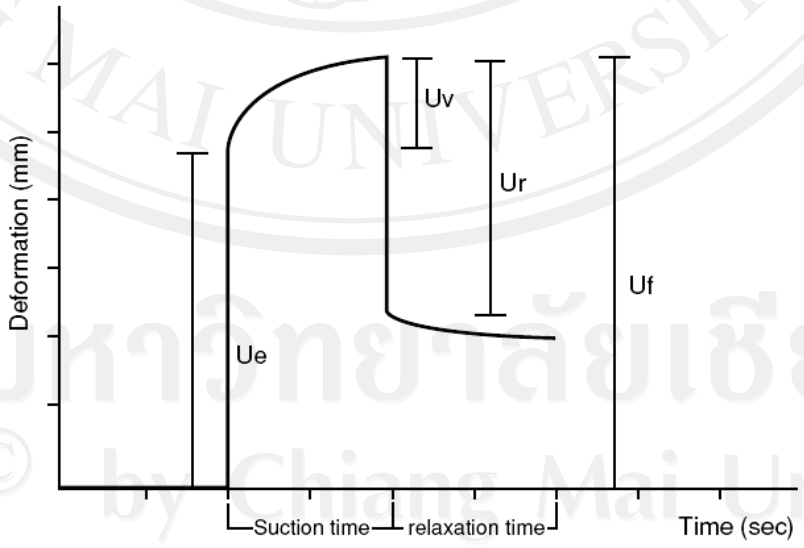


Figure 33 Deformation-time curve of the viscoelasticity of the skin (Dobrev 2002)

B. Skin surface microstructure measurement

Estimation of skin smoothness and wrinkles are of ever increasing interest, especially in the field of cosmetic research. There are some established methods for assessing skin smoothness, for examples, optical and mechanical profilometry. There are direct and non-direct measurements of skin topography. Direct measurements of skin topography are performed by taking the PRIMOS (Prime Computer for its minicomputer systems). The PRIMOS optical three-dimensional *in vivo* skin measurement device is projected onto the skin surface and depicted on the CCD chip of a high-resolution camera (Friedman et al., 2002). Optical profilometry is performed with skin replicas and the detection of the black-and-white reflections by light irradiation caused by different depths and angle of skin furrows. The image processing system consists of a high-resolution black-and-white video or CCD camera and a connected computer using special image processing software (Serup et al., 1995). Laser profilometry is an optical measuring method based on the principle of light amplification and reflection from a replica of skin. A recently developed method is the visiometer technique, which uses a thin silicon print of the skin surface, which allows parallel light to pass through and is registered as a change of transparency by a CCD video camera (Fischer et al., 1999; Hatzis, 2004).

The thickness of the relief is calculated according to the Lambert-Beer law as absorption of the transmitted light. The absorption is measured by the CCD chip and processed into gray levels. These differences are transferred to a monitor, which shows the structure of the skin surface topography. The advantage of this method is the short processing time and the direct visual control of the skin replica

Table 8 Parameters used in the assessment of skin visiometer SV600 (Lee et al., 2008)

Parameter	Description
R1	Skin roughness
R2	Maximum roughness
R3	Average roughness
R4	Smoothness depth
R5	Arithmetic average roughness

on the computer display. The parameters characterizing the cutaneous relief are shown in **Table 8**. All of the parameters values can be used to indicate an increase progressive aging.

C. Skin hydration measurement

Skin hydration is a key factor contributing to skin health. The determination of skin hydration *in vivo* is often made indirectly by measuring some property of the skin that is correlated with the water contents. Two methods commonly used to collect skin moisture information are based on the electrical properties including capacitance and alternating current conductivity. One of these measurements is the determination of skin hydration using a method known as corneometry. This technique determines the capacitance of the skin due to its behavior as a dielectric medium and assesses a 10–20 μm thickness of the SC. The skin hydration values are given in 'arbitrary units' (arb. units) (Blichmann and Serup, 1988).

D. Skin erythema and pigmentation measurements

Various physical, chemical and biological substances cause erythema (redness) and stimulate pigmentation (tanning) in human skin. Mexameter is a

narrow-band reflectance spectrophotometer, and is designed to measure the intensity of erythema and melanin pigmentation (Yoshimura et al., 2001). It has been reported that this instrument is highly discriminative and sensitive enough to detect small differences in skin color (Clarys et al., 2000). This probe relied on the measurements of the reflectance at two to four broad spectral bands corresponding to the output of the light-emitting diodes used for illumination. Due to the spectral overlap of deoxyhaemoglobin (deoxy-Hb) and melanin analysis of reflectance values from a limited number of broad spectral bands, it is often wrought with artifacts, hemoglobin showing a large peak in the green wavelengths (568 nm) with little absorption in the red wavelengths (655 nm), while melanin absorbs light of all wavelengths (Robertson, 1977; Fuchs, 1989).