CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Aging is the accumulation of the changes of the body in a person over time. Skin changes are among the most visible signs of aging. The evidence of increasing age includes wrinkles and sagging skin. Changes in the connective tissue reduce the skin's strength and elasticity. The skin is at higher risk for skin injury with increasing age. The skin is thinner, more fragile, and the protective subcutaneous fat layer is lost. In addition, the ability to sense touch, pressure, vibration, heat and cold may be reduced. Rubbing or pulling on the skin can cause skin tears. Fragile blood vessels are easily broken. Aging skin repairs itself more slowly than younger skin. Anti-aging products that include natural and organic ingredients such as botanical herbs, and minerals are rising in popularity. The global anti-aging products market tends to reach $291.9 billion in 2015, according to the new report by global industry analysts. Hundreds of new products are brought to market 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 expanded exponentially. Consumer concern regarding to harmful skin cancer and wrinkles are rising continually. Alternative medicines, such as herbs appear to be more interesting than drugs or hormones which usually cause side effects. A vast array of compounds is required to supply these demands. Thailand is situated in a hot and humid climatic zone which constitutes a variety of tropical resources, large variety of plants, animal

and microbe species. Hundreds years of Thai traditional medicines have involved in daily life of Thai people. The natural health and beauty care of Thai wisdom has been passed on from generations to generations. At present, natural active ingredients in cosmetics are improved in order to be responsible for modern lifestyle which increases efficiency for Thai herbs and inherits for Thai wisdom.

Thailand is one of the main producers of tropical fruits in the world. Long Kong (*Lansium domesticum* Correa), a member of family Meliaceae, is important as an economic crop as the edible fruits which is widely eaten fresh as dessert. The fruits can also be bottled in syrup. Several parts of the plant are used in traditional medicines. The bitter seeds can be grounded and mixed with water to make a deworming and ulcer medication. The bark is used to treat dysentery and malaria. The powdered bark can also be used to treat scorpion stings. The fruit's peel is used to treat diarrhea, and in the Philippines the dried peel is burned as a mosquito repellent. It has reported that the fruits of *L. domesticum* can also be used in cosmetics with antioxidant property as well as moisturizing and lightening effects with a good safety profile (Tilaar et al., 2007a). Leaf is a part of *L. domesticum* containing tetranortriterpenoids or limonoids as major compounds. There are very few publications concerning the biological activities of limonoids. Sithisarn et al. showed that leaf aqueous extract of Siamese neem tree (*Azadirachta indica A.* Juss var. *siamensis* Valeton) which belongs to the family Meliaceae, exhibited significantly stronger free radical scavenging activities as determined by both DPPH and total antioxidant activity methods (Pongtip et al., 2005). The antioxidants in leaf of *L. domesticum*, will be more value added when developed as cosmetic formulations and more effective and convenient use than the traditional method. In addition, the

development of leaves of *L. domesticum* as anti-aging cosmetic has not been studied. In this study, the natural extract which has biological activities was selected and developed as cosmetic formulations for aging skin treatment. The results from this study will obtain a novel antioxidant from natural extract for the treatment of wrinkle and dull skin which are one of aging skin symtomps.

1.2 Objectives

The purpose of this study was to develop the anti-wrinkle cosmetic formulations containing semi-purified extract from leaves of *L. domesticum*.

1.3 Scope of study

This study was divided into 6 parts which were the followings:

Part 1 : Preparation and *in vitro* anti-aging activities of the crude extracts from the selected part of *L. domesticum*

1.1 Selection of a part of *L. domesticum* which gives the best biological activities

1.2 Preparation of the crude extracts from a part of *L. domesticum* by solvent or aqueous extraction and hot or sonication methods

1.3 *In vitro* anti-aging activities assay of the crude extracts and phytochemical determinations

1.4 Selection of the extracts containing the bioactive compounds which has the highest *in vitro* anti-aging activities

Part 2 : Preparation and *in vitro* anti-aging activities of the semi-purified extracts

2.1 Partial purification of the semi-purified extracts from the selected crude extract by liquid-liquid extraction

2.2 *In vitro* anti-aging activities and phytochemical determination of the prepared semi-purified extracts

2.3 Specification tests of the semi-purified extracts

2.4 Selection of the semi-purified extract which gave the highest *in vitro* anti-aging activities

Part 3 : Discoloration and *in vitro* anti-aging activities of the discolored semi-purified extracts

3.1 Discoloration of the selected semi-purified extract by partition technique

3.2 *In vitro* anti-aging activities and phytochemical determinations of the discolored semi-purified extracts

3.3 Specification tests of the semi-purified extracts

3.4 Selection of the discolored semi-purified extracts which give the highest *in vitro* anti-aging activities and stable for cosmetic formulations

Part 4 : Development of cosmetic base formulations

4.1 Preparation of cosmetic base formulations

4.2 Freeze-thaw cycle for investigation of the physical characteristic of cosmetic base formulations

4.3 Selection of the most stable cosmetic formulations

4.4 Rabbit skin irritation tests of the prepared cosmetic base formulations

4.5 Selection of the best cosmetic base formulations

Part 5 : Development of the cosmetic formulations containing the extract from leaves of *L. domesticum*

5.1 Preparation of cosmetic formulations containing the extract from leaves of Long Kong

5.2 Physico-chemical properties of the prepared cosmetic formulations

5.3 Rabbit skin irritation test of the prepared cosmetic formulations by closed patch test

5.4 Selection of the most stable prepared cosmetic formulations

Part 6 : *In vivo* anti-aging tests of the developed cosmetic formulation containing the extract from leaves of *L. domesticum*

6.1 Preparation of the cosmetic formulations containing the extract from leaves of Long Kong

6.2 Rabbit skin irritation tests of the developed cosmetic formulations

6.3 Performance tests on volunteers of the developed cosmetic formulations

6.4 Satisfactory evaluation of the developed cosmetic formulations

1.4 Literature reviews

1.4.1 Aging skin

Aging changes in the skin are a group of common conditions and developments that occur as people grow older. Skin changes are the most visible signs of aging. Evidence of increasing age includes wrinkles and sagging skin. Whitening or graying of the hair is another obvious sign of aging. With aging, the outer skin layer (epidermis) thins. The pigment-containing cells (melanocytes) decreases, but the remaining melanocytes increase in their sizes. Aging skin appears thinner, more pale, and clear (translucent). Large pigmented spots called age spots, liver spots, or lentigos, appear in sun-exposed areas (**Figure 1**). Changes in the connective tissue reduce the skin's strength and elasticity. This is known as elastosis and especially pronounced in sun-exposed areas (solar elastosis). The blood vessels of the dermis become more fragile. This leads to bruising, bleeding under the skin. Sebaceous glands produce less oil which makes it harder to keep the skin hydration and results in dryness and itchiness. The subcutaneous fat layer becomes thinner and reducing its normal insulation and padding. This increases the risk of skin injury and reduces the ability to maintain body temperature (Minaker, 2007).

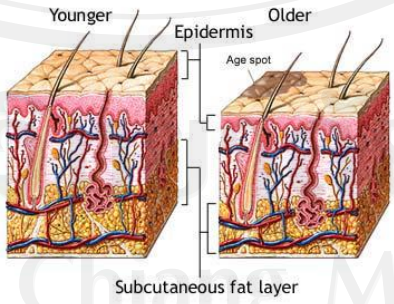


Figure 1 Comparison of the skin cross-sections of the younger and older skin (http://www.cosmeticsurgeryforums.com/images/agedskin.jpg)**.**

1.4.1.1 Cause of aging skin

Lines and wrinkles are influenced by intrinsic and extrinsic factors. The intrinsic (internal) factor is the biological changes which are age, gender, hormone, genetic disposition and race. Biological aging is the result of changes, often genetically determined, that occur naturally within the body. The biological or chronological age can be determined by genetic disposition. Biological aging occurs as a result of natural changes within the body that manifest themselves in visible signs of aging on the skin. Frequently, changes associated with biological aging are the result of a gradual shift in the balance of certain hormones and messenger molecules excreted by other glands and organs within the body. The numerous factors are age, gender, genetic disposition and nationality. Many of these changes are genetically determined and cannot be stopped.

The extrinsic (external) factor acts to prematurely age skin because of the environments such as UV light, weather and climatic influences, nutrition, tobacco and alcohol abuse. The environmental aging occurs as a result of daily exposure to trillions of free radicals from a variety of sources such as the sun's ultraviolet rays, pollution, smoke, harsh weather, tobacco, alcohol abuse, and external stress. These free radicals damage lipids, proteins, and DNA, which limit the ability of cells to function and cripple the integrity of overall cell composition. Mechanical aging is one of the external factors which are continually repeated wrinkle causing behaviors, muscle movements repeated such as repetitive facial expressions, gravity, and sleeping positions. These environmental stress on cellular structures results in the premature aging of the skin (Cosgrove, 2007). The overall qualitative differences between intrinsic and extrinsic aging are summarized in **Table 1**.

Table 1 The comparison between features of intrinsic and extrinsic factors of aging skin (Lavker, 1995)

Features	**Intrinsic aging**	**Extrinsic aging**
Clinical appearance	Smooth, unblemished Loss of elasticity	Nodular, leathery, blotchy Wrinkling, often deep
Skin surface marking	Overall maintenance of normal geometric patterns	Markedly altered and often effaced
Viable epidermis		
Thickness	Thinner than normal	Acanthotic in early stages, atrophy in end stages
Proliferative rate	Lower than normal	
Basal keratinocytes	Modest cellular irregularity	Higher than normal
Keratinization	Unchanged	Marked heterogeneity, numerous dyskeratoses
Stratum corneum	Normal thickness; 'basket-weave' pattern	Unchanged Heterogeneity: 'basket-weave' and compact patterns
Dermo-epidermal junction	Loss of rete pegs. flat: modest reduplication of lamina densa	Loss of rete pegs, flat: extensive reduplication of lamina densa
Dermis		
Grenz zone	Absent	Prominent
Elastin	Elastogenesis. followed by elastolysis 'moth-eaten' fibers	Marked elastogenesis followed by massive degeneration dense accumulations in fibers
Lysozyme	Modest deposition on elastic fibers	Increased deposition on elastic fibers
Collagen	Modest change in bundle size and organization	Moderate change in bundle size
Microvasculature	Normal architecture	Abnormal deposition of basement membrane-like material
Inflammatory cells	No evidence of inflammation	Perivenular, histiocytic-lymphocytic infiltrate

1.4.1.2 Mechanisms of aging skin

With increasing age, the physiology and appearance of the human skin change. Alterations in structure, loss in tightness, smoothness and a decrease in the skin's functional capacity are phenomena which are attributed to the aging mechanisms. An increase in dryness and thus roughness as well as a loss in elasticity and even pigmentation are also a sign of increasing skin aging. Wrinkles on flaccid skin develop with growing age. There is a decline in the subcutaneous fatty tissue. Mature skin is characterized by a decreasing in barrier function, slower metabolic activities in all cells, a strong loss in humidity as well as a decrease in activity of the sebaceous and sweat glands.

Primarily, changes in the skin's appearance are a result of a general aging process of the connective tissue of the subcutis. This leads to an atrophy of the epidermis which adjoins the papillary layer and to an irregular decrease in the elasticity of the elastic nets which are structures accompanying the collagen fibers in the connective tissue. As a result of the changed amount and chemical composition of the basic substance of the connective tissue, a loss of liquids is the result, which consequently leads to a decrease in glycosaminoglycans, the basic structures of the connective and supporting tissues. As a consequence, the youthful turgor such as the skin's tension is lost. Melanocytes disintegrate or lose close contact to epidermal cells and finally lead to a spotted pigmentation of the skin (Leonhardt, 1990).

Figure 2 shows a schematic cross-section through the layers of the different aged skin. The epidermis, also known as corium or horny layer and dermis are represented. In the stratum corneum, the outer visible part of the collagen

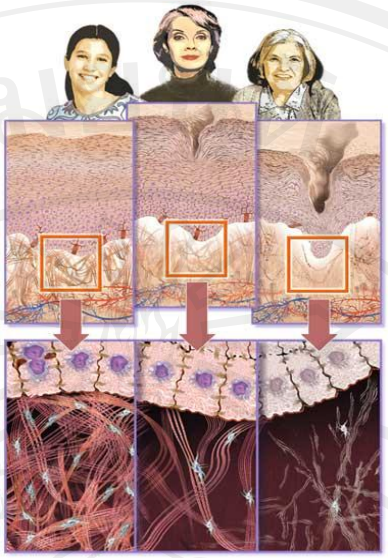


Figure 2 A schematic cross-section through layers of the different aged skin (Leonhardt, 1990)

fibers is represented. This is a typical feature of the dermis. This fiber net is made up of collagen, a protein and main component of intercellular supporting substances, provides the skin with tensile strength and a reversible formability. The dermis which holds blood and lymph vessels, the nervous network, connective tissue cells and cells of defense, is rich in glycosaminoglycans which are in charge of retaining water in the cells. Bundles of collagen fibers which are responsible for the building of tension within the connective tissue show a disorderly alignment. Younger skin shows a more complex network, a crossed and spiraled order of collagen fibers which aging progresses constantly loses its density, firmness and regular alignment than the older skin. Both epidermis which is located over the dermis, and the basal membrane which connects these two skin layers, are affected by aging mechanisms. In the dermis, changes in the composition of matrix proteins are primarily to be attributed to a loss in the firmness and elasticity (Leonhardt, 1990).

In youthful skin, collagen is firm, taut and abundant. In older skin, the collagen structure begins to fall away. They are diminished and fragmented, since the increased production of the enzyme collagenase which breaks down collagen. Then fibroblasts, the critical players in firm, healthy skin, lose their normal stretched state. They collapse, and then more breakdown enzymes are produced which contributes to sag and wrinkle (Fisher et al., 2008).

1.4.1.3 Types of aging skin

Skin wrinkling varies in severity according to various factors such as the number, length and depth of the lines. Typically, gradations in depth are distinguished by categorizing lines or fine lines, wrinkles and folds as shown in **Figure 3**. Fine lines are more superficial and only extend into surface layers (epidermis) of skin while wrinkles are deeper creases into the inner layer of the skin (dermis). Folds are the deepest level creases beneath the skin (subcutis). In general, the least aggressive wrinkle treatments such as topical creams are more effective in improving the appearance of fine lines, while more invasive procedures such as face lifts, laser resurfacing, and injections are required to achieve a similar degree of improvement in the appearance of deeper wrinkles.

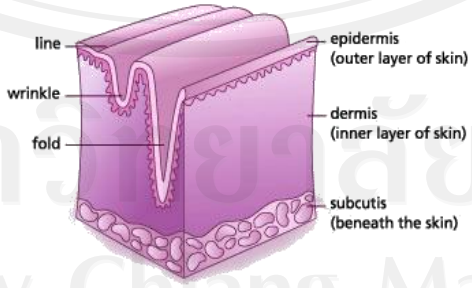


Figure 3 The difference between line, wrinkle and fold of the facial aging skin base on the depth of crevices in each skin layer (http://www.cosmeticsurgeryforums .com/images/depthofwrinkles.jpg)

Procter & Gamble beauty researchers have described types of aging according to their characteristics as fine lines and wrinkles. The definitions have been utilized by the criteria as listed in **Table 2**.

Table 2 Criteria used for distinguish types of aging according to their characteristics by Procter & Gamble beauty researchers (P&G beauty grooming, 2011)

Surface Wrinkles / Fine Lines	Deep Wrinkles / Folds
- Crevices in the surface of the skin that only delve into the epidermal layer - Less than 0.1 mm in depth - Disappear or barely discernible when the relaxation of the skin - Can be reducible/eliminated without the use of invasive procedures such as moisturizer, anti-wrinkle cream	- Crevices in the skin surface that delve into the dermal layer (deep wrinkles) and even into the subcutaneous layer (folds) - Greater than 0.1 mm in depth and up to 0.4 mm in depth - Well-defined and evident when the relaxation of the muscles and the skin - Difficult to reducible/eliminated

Histopathological examination can also reveal the presence of lesions in all types of aging skin involving the epidermis, corium, and subcutaneous tissue. The dermis of the healthy young skin is characterized by thick, wavy bundles of collagen with little space in between the bundles. Interstitial cells imbedded in the connective tissue are in intimate contact with collagen fibers and have a flattened or spread appearance. Nuclei tend to be large, oblong and light-staining. In contrast, the collagen fiber bundles are shorter and thinner in the aged skin and have more space between the fiber bundles. The collagen bundles have a disorganized appearance with

tangled fibers running in all directions. Cells imbedded in the collagen matrix have a rounded appearance and many of the cells appear to be separated from the surrounding collagen. The biochemical evidence directly increases the damage of collagens in both healthy young and old skin. The biochemical data are based on assays that distinguish undamaged collagen from its fragmented counterpart by the fact that undamaged collagen is resistant to digestion by serine proteinases while fragmented collagen can be further degraded to small peptides and single amino acids. The chymotrypsin sensitivity of dermal collagen in the healthy young skin versus old skin has been compared. The amount of the fragmented collagen in the old skin is increased by approximately four-fold over the amount present in healthy skin. The total collagen is decreased by 20–30% in the skin of aged (80+ years) individuals as compared to the young skin of individuals aged 18–29 years.

Figure 4 compares the histological features seen in the sun protected skin of a young adult and the changes that occur as a consequence of the chronological aging process. In the skin from a young adult (a), thick collagen fiber bundles are present throughout the dermis, extending almost to the epidermis. Interstitial cells imbedded in the collagen have a stretched shape and appear to be in intimate contact with collagen. In the skin sample from the old individual (b), healthy collagen bundles have been replaced with thin, short, disorganized fibers. More open space appears in the dermis. Interstitial cells are round or oblong, and some appear to be surrounded by open space. The epidermal layer is similar in skin from young and old individuals. However, the epidermis is thin in old skin and exhibits a loss of rete ridges and rete pegs. Photodamaged skin (c) shows extensive collagen damage as in old skin but it is difficult to detect due to the build-up of elastotic material (Varani et al., 2011).

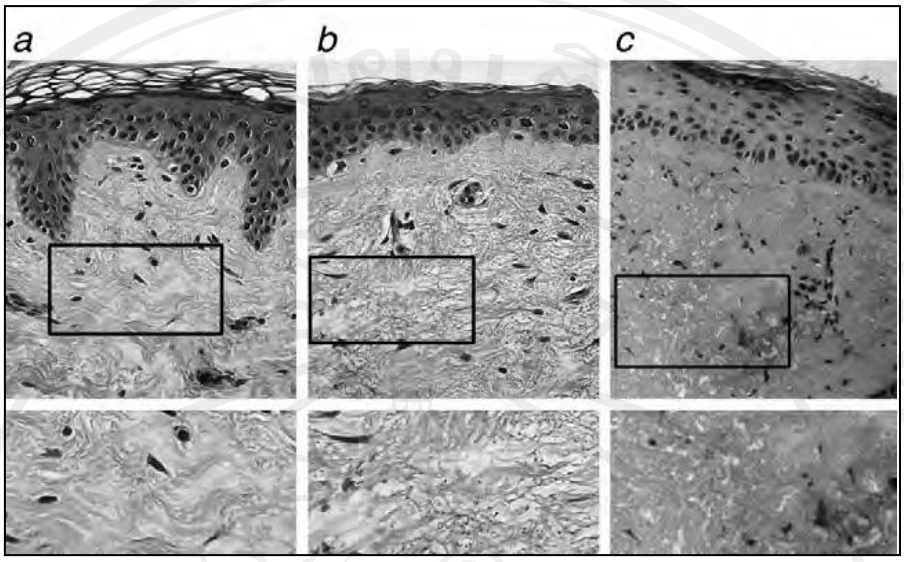


Figure 4 Histological features of the healthy young skin (a), old skin (b) and photodamaged aged skin (c) Upper panels are 5 mm hematoxylin and eosin-stained sections (X160). Lower panels (X240) (Varani et al., 2011)

1.4.1.4 Treatments of the aging skin

There are two treatments of the aging skin as the followings:

A. Medical treatments

Chemical peels are one of the most popular choices for both patient and physician. In comparison to the newer options available, it has a long- standing safety, efficacy, low cost and quick recovery time. Various acidic and basic compounds are used to produce a controlled skin injury. They are classified as superficial, medium-depth, and deep peeling agents according to their level of penetration, destruction, and inflammation. In general, superficial peels cause epidermal injury and occasionally extend into the papillary dermis. Medium-depth peels cause injury through the papillary dermis to the upper reticular dermis. Deep peels cause injury to the mid-reticular dermis (Stegman, 1982). The effect of a

chemical peel is dependent on the agent used, its concentration, and the techniques employed before and during its application (Monheit et al., 2001). The examples of chemical reagent used in peeling such as alpha-hydroxy acid (such as glycolic acid, lactic acid, malic acid, citric acid and tartaric acid), trichloroacetic acid, tretinoin and salicylic acid.

Wrinkle fillers are medical device implants approved for medical use to fill in facial wrinkles in order to create a smoother appearance or structural augmentation of the lips, lines, folds, and wrinkles (**Figure 5**). Various substances have been injected into the face, including wax, silicone, and animal products. When injecting the facial skin, most filler are placed intradermally. With the many choices of filler types and particle sizes, there are over 150 injectable fillers on the global market, but only a dozen have FDA approval (U.S. Food and Drug Administration, 2010). Fillers can be permanent or temporary and classified into three categories including collagens, hyaluronic acids and biosynthetic polymers. The most popular injectable locations are smile lines, nasolabial folds, perioral lines, periorbital lines, glabellar lines, forehead lines and oral commissures (**Figure 5**).

Laser resurfacing is a technique used in the laser surgery aim to dissolves the molecular bonds of a material at the desired area. Laser stands for Light Amplification by the Stimulated Emission of Radiation. The laser beam can gently vaporize and/or ablate skin tissue to improve wrinkles, scars and blemishes, seal blood vessels or cut skin tissue, solar lentigenes, sun damage, stretch marks, actinic keratosis and telangiectasias. The laser also has the unique ability to produce one specific color (wavelength) of light, which can be varied in its intensity and pulse

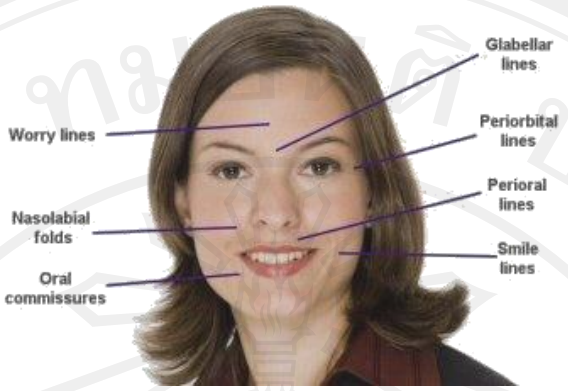


Figure 5 The major injectable locations of the facial dermal filler treatments (http://www.skinetc.co.uk/Portals/13/Filler.jpg)

duration. Two types of lasers are used for cosmetic skin treatments that are ablative and non-ablative lasers. The ablative laser removes a layer of skin that the new skin can flourish and fill in the wrinkles, but heating from the laser can create open wound. In the contrast, the non-ablative devices are great for mild to moderate wrinkling and photoaging, and fairly eliminate most acne scarring treatments that are only a little uncomfortable followed by roughly of redness (Alster, 1999; Alster et al., 2004). The laser beams utilized for aging skin treatment at various wavelengths were listed in **Table 3**.

Botox or botulinum exotoxin is a naturally occurring neurotoxin produced by the anaerobic gram-positive *Clostridium botulinum* bacterium. Only botulinum toxin type A is currently approved by the U.S. Food and Drug Administration (FDA) for cosmetic use. The mechanism of botox is to prevent the release of acetylcholine at the motor end plate. Without this release, the electrical impulse is not transmitted; hence, the muscle does not move. Selective paralysis is the major neuromodulator treatment when the toxin is in the anticipated and desired location. Botox is

Table 3 The laser beam used for aging skin treatments (American National Standards Institute, 2005)

Laser types	Typical dermatological uses	Wavelength (nm)
Argon	Blood vessel reduction (treatment of vascular lesions)	488, 514
Copper Vapor	Treatment of vascular lesions (treatment of telangiectasia)	510, 578
Nd: YAG and Nd: YAG-KTP	Tattoo pigmentation breakup and blood vessel reduction (treatment of vascular lesions such as telangiectasia blemishes)	532, 1064
Diode	Blood vessel photocoagulation (treatment of vascular lesions)	800-820
CO_2	Skin Resurfacing	10, 600

Note: Nd: YAG represents neodymium:yttrium-aluminum-garnet and Nd: YAG-KTP represents neodymium:yttrium-aluminum-garnet potassium titanyl phosphate

with known allergy to botox or human albumin, neurological disorders such as myasthenia gravis, multiple sclerosis, and Eaton-Lambert syndrome. Aminoglycoside class of antibiotics may interfere with neuromuscular transmission, threrfore, the botox injections in patients taking aminoglycosides must avoid or delayed (Vartanian et al., 2004).

Facial surgery is a surgical method that removes excess facial and neck skin to make the face and neck appear younger. The facial surgery including microdermabrasion which is a micro needle device used to smooth out fine facial wrinkles, does not arrest the aging process. The surgery helps to enhance the skin's structure by lifting the skin and removing the fat in specific areas. Additional procedures such as forehead lift, liposuction, eyelid lift or rhinoplasty, necessary to support the face and neck lift surgery for ensuring the best results (Goin et al., 1989). The surgery is an extremely effective procedure to tighten excessive hanging skin. It also has some risks such as bleeding, infection, scarring, damage to deeper structures,

asymmetry, surgical anesthesia, nerve injury, chronic pain, skin cancer, unsatisfactory results, allergic reactions, hair loss, delayed healing, long-term effects and required additional surgery (Grossbart et al., 1999).

B. Cosmeceutical treatment

Antioxidants is one of several approaches to diminish oxidative injury in the skin for cosmetic and cosmeceutical applications. The external application could supplement to supply the antioxidants preventing formation and proliferation of ROS. Ascorbic acid (vitamin C), α-tocopherol (vitamin E), isoflavones and polyphenols are examples of antioxidant active ingredients used in cosmetics. Additionally, plant extracts which is a rich source of antioxidant compounds have become popular. There have been several publish investigations studied on the *in vitro* and *in vivo* evaluation of anti-aging properties of these antioxidants from natural extracts. The semi-purified extract from *Terminalia chebula* Retz. gall, consisted of gallic acid as a major compound, exhibited high *in vitro* antioxidant activity including DPPH radical scavenging and metal chelating activity. For *in vitro* anti-wrinkle assessments in human volunteers, the cosmetic formulations containing the semi-purified extract of *T. chebula* gall which loaded in niosome demonstrated significantly improvement of skin elastic and roughness (Manosroi et al., 2011).

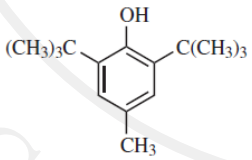
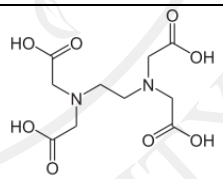
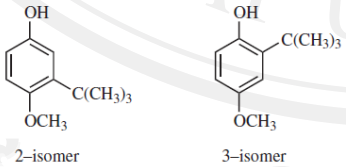
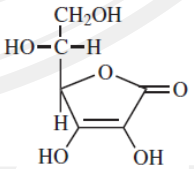
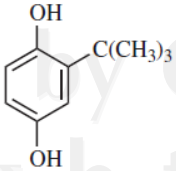
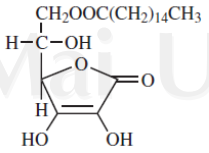
1.4.2 Anti-aging agents/products

1.4.2.1 Synthetic antioxidants

There are a large number of synthetic compounds that have been shown to have anti-aging properties. Many compounds have antioxidant

functions or alter the collagen metabolism. There are, however, also new compounds that have a completely different mode of action to reduce aging or wrinkle formation (Trueb, 2005). **Table 4** demonstrated some of the synthetic antioxidants used in cosmetic formulas which are carcinogenic, for example; 2,6-ditert- butyl-p-hydroxy toluene (BHT), butylated hydroxyanisole (BHA) (Singh et al., 2010), propyl, octyl and dodecyl gallate and tert-butylhydroquinone (TBHQ), hence, the extensive use of these raw materials in cosmetics may represent a potential health risk (Hirose et al., 1997; Ito et al., 1986). Long term and widespread studies indicate that the use of

Table 4 The chemical structures of synthetic antioxidants used in cosmetics (Shahidi et al., 2005)

The chemical structure of synthetic antioxidants	
	
Butylated hydroxytoluene (BHT)	Ethylenediaminetetraacetic acid (EDTA)
	
Butylated hydroxyanisole (BHA)	Erythorbic acid
	
Tert-butylhydroquinone (TBHQ)	Ascorbyl palmitate

synthetic antioxidants in cosmetic products can result in potential health risks associated with their intake (Tsai et al., 2008). A particular case in these sense can be represented by the category of make-up cosmetics, like lipsticks (Maw-Rong et al., 2006).

1.4.2.2 Natural antioxidants

Because of side effects of synthetic antioxidants, a large number of researches on natural sources of antioxidants are continuously increased. Natural antioxidants bring less rigorous burden of safety proof than that required for synthetic products. However, natural antioxidants may possess several drawbacks, including high usage levels, low antioxidant efficiency, undesirable flavor or odor, and possible loss during processing. The safety of natural antioxidants can not be taken for granted if have potential mutagenicity, carcinogenicity, teratogenicity, or other pathogenic activities, for example, renal and hepatotoxicity for chronic use of nordihydroguariaretic acid (NDGA) which is a natural constituent of *Larrea divaricata* (DC.) Coville (Reische et al., 1998; Shahidi et al., 1992) (**Figure 6**).

L-ascorbic acid and tocopherols are the most important commercial natural antioxidants. In addition, many naturally occurring including phenolic, flavonoid, triterpenoid anthraquinone, carotenoid and saponin antioxidants have been identified in plant sources and vegetable extracts that is applied in anti- aging cosmetic applications (Shahidi et al., 1992). Recent research has focused on isolation and identification of effective antioxidants of natural origin (Reische et al., 1998). Polyphenol compounds are widely studied for their antioxidant properties and were developed as active ingredients for anti-aging cosmetic, especially, green tea and black tea leaves extracts (*Camellia sinensis* Linn.) containing tannins as major

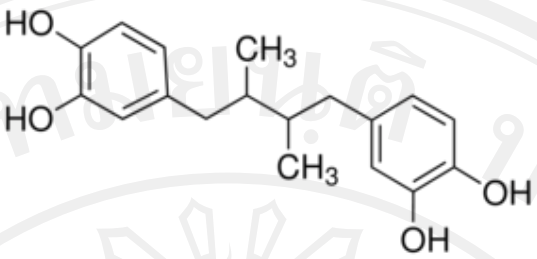


Figure 6 Chemical structure of the nordihydroguariaretic acid (NDGA), a natural antioxidants extracted from leaves of *L. divaricata* which were used in drugs and cometics (Vasek, 1980)

antioxidants such as catechin, epicatechin, epigallocatechin and epicatechin gallate, etc. Flavonoids are also well-known antioxidants in plants. A class of flavonoids called anthocyanins and anthocyanidins provides colors of fruits and flowers. These compounds are capable of increasing the intracellular levels of vitamin c, improving capillary stability, inhibiting the destruction of collagen, and inactivating microbial enzymes used to spread infection (Wada et al., 2002). Nowadays, the most important botanicals pertaining to cosmeceutical use include teas, soy, pomegranate, date, grape seed, pycnogenol, horse chestnut, chamomile, curcumin, comfrey, allantoin, and aloe (Glaser, 2004). **Table 5** showed examples of anti-aging extracts from natural resources, their active antioxidant compounds and relative action on anti-aging process of skin.

Table 5 Anti-aging compounds from natural resources (Glaser, 2004; Trueb, 2005)

Active Ingredient	Compound/Origin	Mode of Action
• Grape Seed Extract	Polyphenols & flavonoids from fruits extracts	Antioxidant
• Collagen, Hydrolyzed	Protein & protein fragments, animal/vegetable derived	Moisturizing, regenerating, anti-wrinkle
• Jojoba Protein	Protein, derived from Jojoba plant	Moisturizing, regenerating, repairing, anti-wrinkle
• Elastin	Protein, animal/plant/marine derived	Moisturizing, restructurating, anti-wrinkle
• Gelatin	Protein, animal/plant/marine derived	Moisturizing, restructurating, anti-wrinkle
• Chondroitin Sulphate	Polysaccharide, animal/plant/marine derived	Moisturizing, regenerating
• Oligopeptides	Oligopeptides, animal/plant/marine derived, synthetic	Stimulates collagen synthesis & skin cells growth (fibroblasts)
• Phytic Acid	Botanical extract	
• Spirulina Extract	Botanical extract from plankton	Antioxidant & chelating agent, scavenges free-radicals
• Calcium PCA	Mineral	Antioxidant, immune-stimulating, moisturizing
• Ceramides	Ceramides packed in milk-lipsosomes (lacto-ceramides)	Stimulates cell differentiation & synthesis of epidermal lipids
• Zea Mays Kernel Extract	Botanical extract from Zea Mays Corn (rich in inositol)	Replenishes own skin-ceramides, recovers barrier function
• DHEA	3-Beta-Hydroxy-5-androsten-17-one	Antioxidant
• Pullulan	Natural sugar (glucan) from fungus A. pullulans	Protective, regenerating
• Ferulic Acid	Phenolic compound, plant-derived	Skin tightening effect, stimulates collagen synthesis
• Hyaluronic Acid	Polysaccharide, derived from soy peptone & yeast extract	Antioxidant, anti-inflammatory Moisturizing, promotes growth of skin cells, anti-wrinkle
• Genistein	Isoflavone compound, plant-derived (e.g. soya, tofu)	Antioxidant, protective
• Kojic Acid Dipalmitate	Natural product derived from Japanese mushroom	Toning, invogorating, skin-lightening/whitening
• Phyllanthus Emblica	Polyphenols & flavonoids from fruit extract	Antioxidant, skin-lightening/whitening
• Coenzyme Q10	Ubiquinone (vitamin-like compound), plant-derived	Antioxidant, promotes collagen & elastin synthesis
• Ectoin	Natural compound derived from halophilic bacteria	Skin-relaxing, moisturizing, protects from cell damage
• TIMP2	Protein, biotechnologically produced	Inhibits enzymes that degrade collagen in the skin
• L-ascorbic acid (Vitamin C)	Natural derived or synthetic	Antioxidant, stimulates collagen synthesis, skin-lightening
• Argireline	Peptide (acetyl hexa-peptide-3)	Anti-wrinkle (inhibits facial skin muscle tightening)
• Dipalmitoyl Hydroxyproline	Amino acid combined with palmitic acid	Contracts collagen, moisturizing, inhibits “age enzymes”
• Retinol Palmitate (Vitamin A)	Natural vitamin, synthetically produced	Regenerating (promotes skin cell growth), anti-wrinkle
• Provitamin B5	Natural vitamin, synthetically produced	Moisturizing, improves skin elasticity, regenerating

1.4.2.3 Anti-aging products in the markets

The global market for anti-aging products for topical, dietary and pharmaceutical is predicted to increase due to growing interest from ever younger consumers. The anti-aging market is projected to reach $274.5 billion by 2013. Asia-Pacific and North America represent the largest growth geographies as demonstrated in **Table 6**.

Table 6 The global anti-aging market growth rate (Global Industry Analysts, Inc., 2008).

Market	2-year projected growth rate				7-year projected growth rate
	2009	2011	2013	2015	
United Stateds: $ Billions	34,848.88	42,207.74	50,626.82	58,734.66	
United Stateds: % Growth	19.17%	21.12%	19.95%	16.01%	76.25%
Europe: $ Billions	31,707.54	37,932.44	44,761.99	52,308.57	
Europe: % Growth	18.94%	19.63%	18.00%	16.86%	73.43%
Japan: $ Billion	11,389.28	13,555.79	15,965.13	18,556.37	
Japan: % Growth	19.30%	19.02%	17.77%	16.23%	72.33%
Asia-Pacific: $ Billions	9,322.09	11,221.25	13,566.91	16,444.23	
Asia-Pacific: % Growth	19.72%	20.37%	20.90%	21.21%	82.20%

According to the increasing of natural lovers, the natural active ingredients become an important factor as consumers begin to seek. These cosmetics claim to anti-wrinkle, anti-aging and facial firming. Examples of the cosmetics containing natural anti-aging active ingredients were launched in the markets in order to supply the demand of anti-wrinkle customers as shown in **Table 7**.

Table 7 Examples of anti-aging or wrinkle products in the markets

Name of product (Manufacturer)	Active ingredient	Product appearance
Avotone anti wrinkle relaxant cream (Natures Pharmacy)	Avocado oil	 http://www.exit15.com/avotone-anti-wrinkle-cream-3-pack-pi-655.jpg
Apivita wine elixir anti-wrinkle and firming night cream (APIVITA N. America)	Red wine, neroli, patchouli, clary sage and geranium essential oils	 http://beautytidbits.com/wp-content/uploads/2012/05/Apivita-Wine-Elixir-Anti-wrinkle-and-Firming-Night-Cream-.jpg
Anti wrinkle cream with clay, ginseng & collagen (Farmec S.A. Cluj Napoca)	Vitamin A, Vitamin E, Vitamin C, Ferulic Acid with Avocado Oil, Wheat Germs Extract, Marigold Extract and Natural Special Clay	 http://www.gerovitalgh3.com/index.php?main_page=popup_image.jpg
Wrinkles Fade with Complexion MD Anti-Wrinkle Cream (Complexion MD)	Pumice, sunflower oil and green tea leaves extract	 http://www.complexionmd.com/images/antiwrinkle.png
Youthtopia™ Lift Firming Cream (Origins Natural Resources, Inc)	Guggul extract (*Commiphora wightii* (Arn.) Bhandari)	 http://women.mthai.com/wp-content/uploads/2011/12/Origins-Firming-Cream.jpg

1.4.3 Long Kong (*Lansium domesticum* Correa)

Lansium domesticum Correa is a genus of small trees from the family Meliaceae found both in the wild and cultivated in Thailand and surrounding countries in Southern Asia. The seeds of the fruit, traditionally, are said to be toxic to the malaria parasite (Leaman, 1995). The fruit is known locally, depending on variety, as *langsat*, *long-kong*, *duku*, *lang-sook*, and *langsat-khao* (in Thai). The phytochemical studies on *L. domesticum* Corr. have presented the isolations of several types of triterpenoids (Habaguchi et al., 1968; Kiang et al., 1967; Nishizawa et al., 1985; Nishizawa et al., 1984; Nishizawa et al., 1982; Nishizawa et al., 1983; Tanaka et al., 2002).

1.4.3.1 Botanical characteristics

L. domesticum Corr. originates in western South-East Asia, from Peninsular Thailand in the west to Borneo in the east (Indonesia). It still occurs wild or naturalized in this area and is one of the major cultivated fruits. The appearance of each part of *L. domesticum* were shown in **Figure 7**. The tree is up to 30 m tall, fluted with steep buttresses; bark mottled grey or orange and furrowed. Its bark contains milky, sticky resinous sap and twigs glabrous. Leaves are alternately, and translucent white aril. The cells without developed seed are also filled with aril tissue (Heyne, 1987; Verheij et al., 1992).

The detection of apomixes in *L. domesticum* Corr. (Khongsuwan, 2005) has been revealed. The detection was carried out by cytoembryological method and RAPD (Random Amplified Polymorphic DNA) technique. Flower, leaf and seed samples of Long Kong, Langsat and Duku were collected and examined for cytoembryological analysis. At the receptive stage multiple embryo sacs in the same

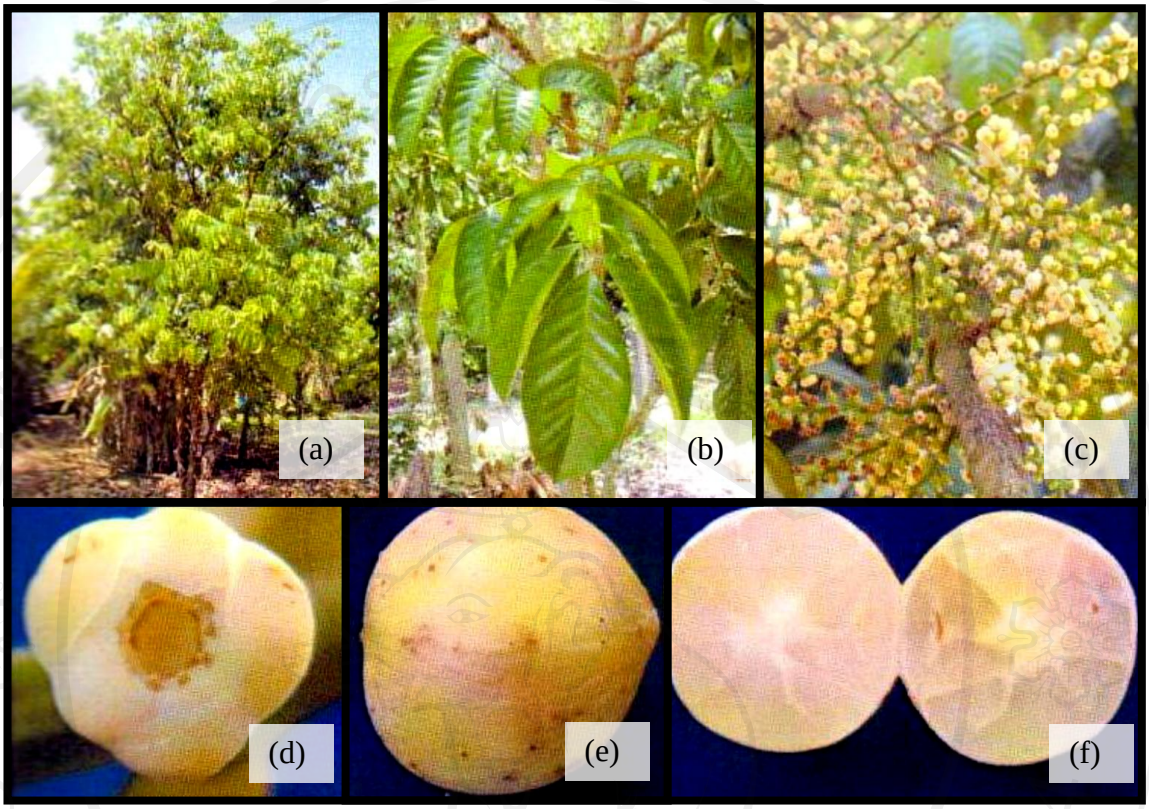


Figure 7 The appearance of tree (a), leaves (b), flowers (c), young fruit (d), ripening fruit (e) and seeds (f) of *L. domesticum* Corr. (Heyne, 1987; Verheij et al., 1992).

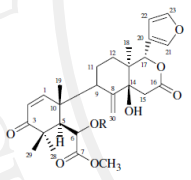
ovule were found in Long Kong and Langsat indicated aposporous apomixes. However, multiple embryosac was not detected in Duku. DNA patterns of Long Kong, Langsat and Duku seedlings including their maternal parents were revealed by RAPD with the following eight primers including OPA-10 (GTGATCGCAG), OPB-04 (GGACTGGAGT), OPB-07 (GGTGACGCAG), OPC-04 (CCGCATCTAC), OPC-05 (GATGACCGCC), OPD-03 (GTCGCCGTCA), OPT-01 (GGGCCACTCA) and OPT-08 (AACGGCGACA). Of total 149 Long Kong seedlings from different 3 maternal plants exhibited identical DNA fragment profiles. It was concluded that all Long Kong seedlings from the study derived from somatic cells and confirmed

obligate apomixes. Based on the RAPD patterns obtained with 8 primers from the study, indicating Long Kong can be propagated true-to-type by seed.

1.4.3.2 Bioactive constituents

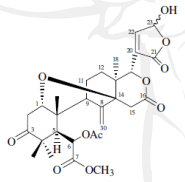
The bioactive constituents of *L. domesticum* Corr. have been isolated and identified continuously. However, some parts of the plant have been investigated and identified chemical structure including seeds, fruit peels, leaves and edible fruits.

Seed of *L. domesticum* is a rich source of six classes of the limonoids and five tetranorterpenoids called domesticulide as shown in **Figure 8**.

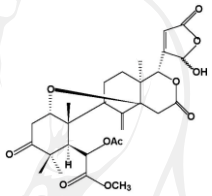


domesticulide A, R = H

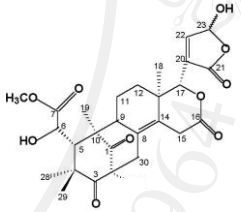
domesticulide B, R = Ac



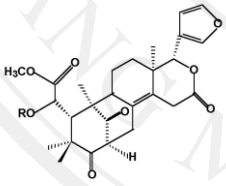
domesticulide C



domesticulide D

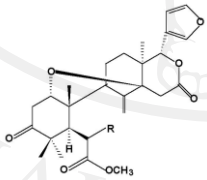


domesticulide E



6-hydroxymexicanolide, R = H

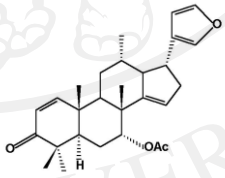
6-acetoxymexicanolide, R = Ac



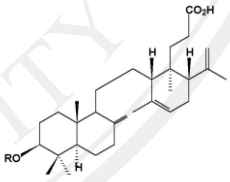
methyl angolensate, R = H

methyl 6-hydroxyangolensate, R = OH

methyl 6-acetoxyangolensate, R = OAc

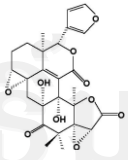


azadiradione

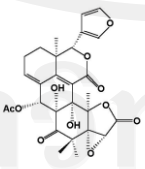


lansiolic acid , R = H

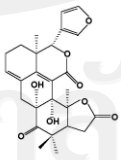
lansioside B, R = glu



dukunolide B



dukunolide C



dukunolide D

Figure 8 Chemical structures of the compounds exist in the seeds of *L. domesticum* (Connolly et al., 1968; Lee et al., 1988; Nishizawa et al., 1985; Nishizawa et al., 1983; Okorie et al., 1968; Saewan et al., 2006)

The elucidated compounds have moderate potential anti-malarial activity against *Plasmodium falciparum* (Saewan et al., 2006). The major dukunolides which are dukunolides A, B and C and the minor constituents were dukunolides D, E, and F were elucidated by colum chromatography from seed of *L. domesticum* (Nishizawa et al., 1985; Nishizawa et al., 1985; Nishizawa et al., 1988) (**Figure 9**).

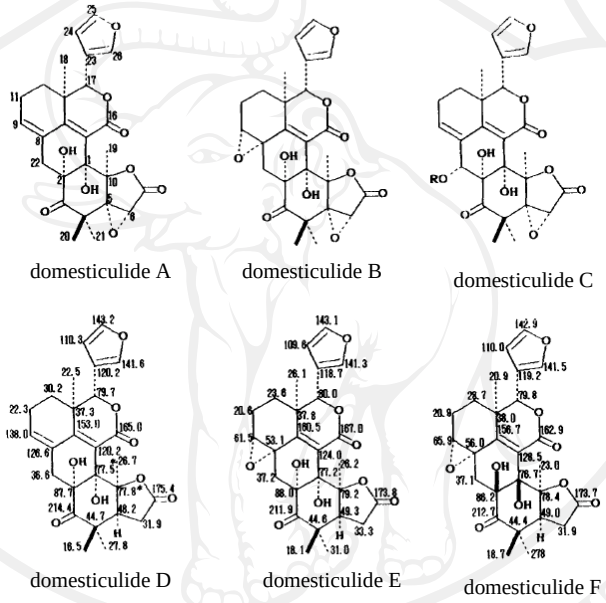
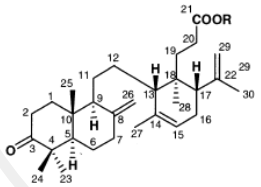


Figure 9 The structure of tetranortriterpenoids found in seed of *L. domesticum* (Nishizawa et al., 1988)

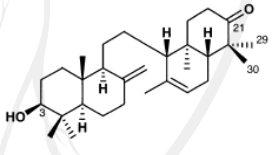
The fruit peels of *L. domesticum*, collected in Thailand which were extracted with methanol showed toxicity against *Artemia salina* (Omuru et al., 1999). The extract was subjected to solvent partitioning and repeated chromatographies to give three triterpenoids together with two known compounds which are lansic acid and methyl lansionate (Nishizawa et al., 1985; Nishizawa et al., 1982; Nishizawa et al., 1983) as illustrated in **Figure 10**. The elucidated compounds named 3*β*-

hydroxyonocera-8(26),14-dien-21-one and 21α-hydroxyonocera-8(26),14-dien-3-one were previously reported as synthetic intermediates for chemical synthesis of lansic acid from α,γ-onoceradienedione (Nishizawa et al., 1986).

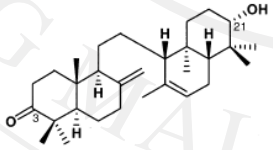
The peel of *L. domesticum* contained seco-onoceranoids which were lansic acid as a major component, and lansiolic acid as a minor component (**Figure 10**) (Nishizawa et al., 1989). The ethanolic extract from fruit peel of *L. domesticum* contains a large quantity of latex. The major constituent of the extract was lansic acid. Lansioside A and its derivatives were some examples of a polar compound found in the fruit peels of *L. domesticum* (**Figure 11**).



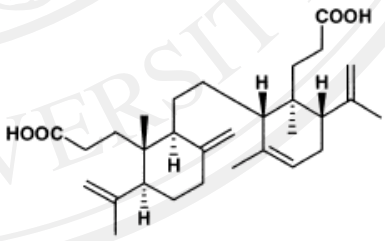
lansionic acid, R = H
methyl ester, R = CH_3



3*β*-hydroxyonocera-8(26),14-dien-21-one



21α-hydroxyonocera-8(26),14-dien-3-one



lansic acid

Figure 10 The chemical structure of onoceranoid triterpenes were isolated from the fruit peels of *L. domesticum* together with two known triterpenoids (methyl lasionate and lansic acid which exhibited mild toxicity against brine shrimp (*Artemia salina*) (Tanaka et al., 2002)

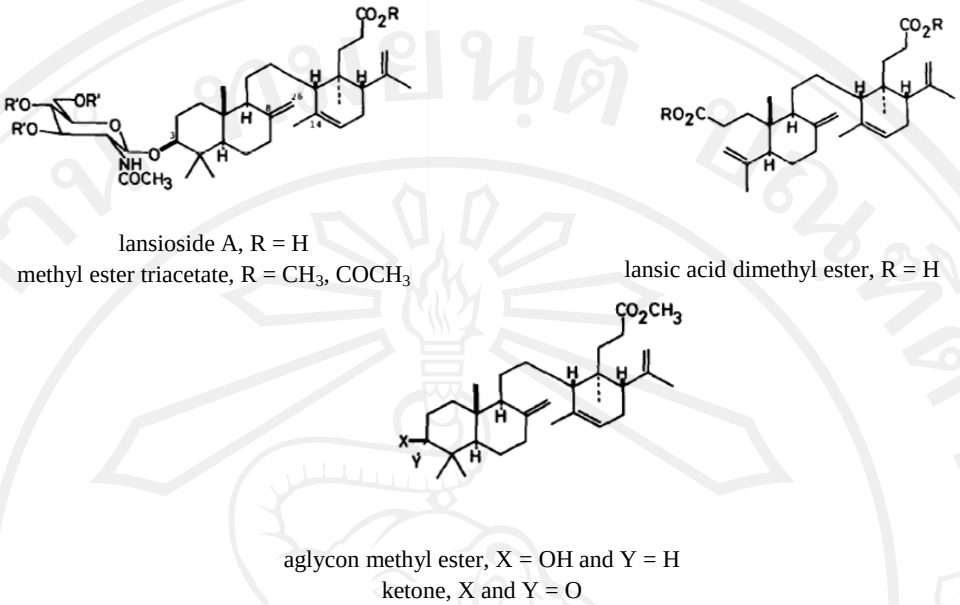
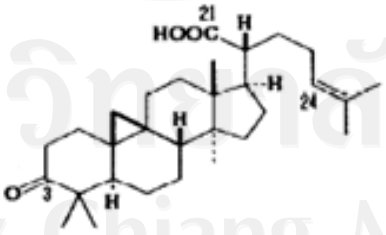


Figure 11 The chemical structure of lansioside A and its derivatives obtained from dry peel ethanolic extract of *L.domestiucm* (Nishizawa et al., 1982)

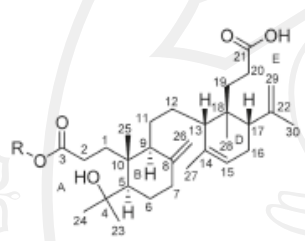
The leaves of *L. domesticum* contained cycloartanoid triterpene, named 3-oxo-24-cycioarten-21-oic acid (**Figure 12**) have been reported. Lansiolic acid was found as a major constituent (**Figure 8**). Some derivatives of 3-oxo-24-cycioarten-21-oic acid showed significant inhibitory activity of skin tumor promotion on the basis of Epstein-Barr virus associated early antigen examination (Nishizawa et al., 1989).



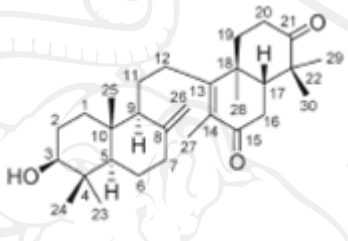
3-oxo-24-cycloarten-21-oic acid

Figure 12 The chemical structure of 3-oxo-24-cycloarten-21-oic which is a minor constituent of leaves of *L. domesticum* (Nishizawa et al., 1989)

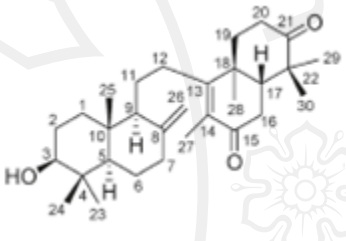
Twigs of *L. domesticum* were extracted and the extract was separated by using column chromatography. A rare class of onoceranoid triterpenoids, represented by lamesticumin A, the ethanolysis product of lamesticumin A, lamesticumins B – F , lansic acid 3-ethyl ester, and ethyl lansiolate, along with four known analogues were isolated from the twigs of *L. domesticum* as shown in **Figure 13** (Dong et al., 2011).



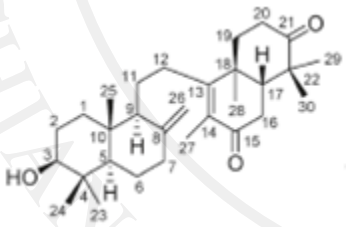
lamesticumin A and the ethanolysis product of lamesticumin A



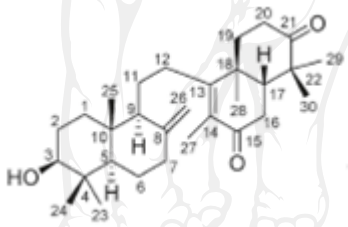
lamesticumin B



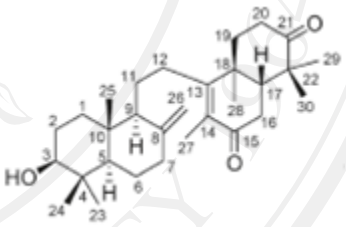
lamesticumin C



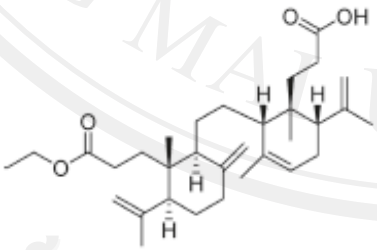
lamesticumin D



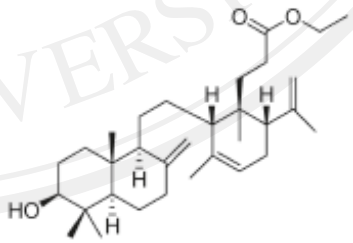
lamesticumin E



lamesticumin E



lansic acid 3-ethyl ester



ethyl lansiolate

Figure 13 Nine new onoceranoid type triterpenoids were isolated from the ethanolic extract of twigs of *L. domesticum* (Dong et al., 2011).

The essential oil and organic acid from dried pulp of *L. domesticum* (Chairgulprasert et al., 2006). The obtained oil was pale yellow with aromatic smell.

According to GC-MS analysis, 20 compounds were identified. The relative percentages of these compounds are recorded in **Table 8**.

Table 8 Chemical constituents of the essential oil from pulp of *L. domesticum* (Chairgulprasert et al., 2006)

Peank No.	Compounds	% of Total
1	L-Linalool	0.80
2	α -Copaene	11.15
3	2,4-diisopropenyl-1-methyl-1-vinylcyclohexane	1.25
4	β-Caryophyllene	1.46
5	γ-Muurolene	4.56
6	Germacrene-D	9.16
7	α -Muurolene	3.69
8	γ-Cadinene	2.42
9	δ-Cadiene	6.74
10	α-Calacorene	2.02
11	(+)-Spathulenol	5.72
12	Aromadenedrene	2.84
13	Ledane	4.09
14	Fonenol	2.05
15	α-Longipinene	1.56
16	Torreyol	4.58
17	τ-Muurolol	6.34
18	(2,3,4-Trimethylphenyl)-2-propanone	2.12
19	Palmitic acid	5.49
20	*Cis*-Oleic acid	14.80

The main constituents were oleic acid, α-copaene, germacrene-D, δ-cadinene, τ-muurolol, (+) spathulenol and palmitic acid. Most of the essential oil components were sesquiterpenes and only one monoterpene, linalool was presented. In addition, organic acids which are maleic acid, citric acid, malic acid and glycolic acid, were detected as shown in **Table 9**.

Table 9 Peak areas and quantities of organic acids in pulp of *L. domesticum* (Chairgulprasert et al., 2006)

Organic acids	Peak area	Quantities of acids (% w/w of dried pulp)
Maleic acid	157938.67	1.23
Citric acid	47915.00	0.22
Malic acid	11878.33	0.15
Glycolid acid	10335.67	0.14

1.4.3.3 Applications and researches on Long Kong

A. Pharmaceuticals

The pharmaceutical properties including anti-malarial, anti-cancer and anti-bacterial activities of various compounds from several parts of *L. domesticum* have been investigated. The anti-malarial activity of the extract from seeds of *L. domesticum* has been reported (Saewan et al., 2006). The sixteen isolated from seeds were evaluated for their anti-malarial activity against *P. falciparum* compared with artemisinin (positive control). The inhibitory concentration (IC_{50}) which has represented the concentration that causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*, were determined. The results from the study demonstrated that, among all of the isolates, domesticulide B, domesticulide C, domesticulide D, 6-acetoxymexicanolide, methyl angolensate, methyl 6-acetoxyangolensate, azadiradione and dukunolide C, have the moderate potential anti-malarial activity with the IC_{50} values of 2.49.7 μg/ml as shown in **Table 10**.

Table 10 Anti-malarial activity of natural compounds isolates from seeds of *L. domesticum* (Saewan et al., 2006)

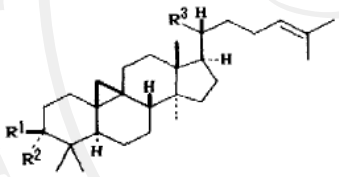
Compounds	**Anti-malarial IC_{50} (µg/ml)**
domesticulide B	3.2
domesticulide C	2.4
domesticulide D	6.9
6-acetoxymexicanolide	9.7
methyl angolensate	5.9
methyl 6-acetoxyangolensate	3.8
azadiradione	2.9
dukunolide C	5.2
Artemisinin	0.001–0.003

Note: IC_{50} means the inhibitory concentration which has represented the concentration that causes 50% reduction in parasite growth

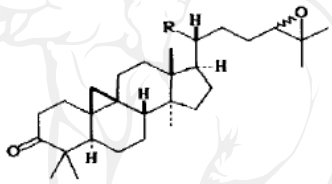
The anti-cancer activity of the natural extract from leaves of *L. domesticum* containing 3-oxo-24-cycloarten-21-oic acid and its derivatives was performed by using *in vitro* assay of the Epstein-Barr virus associated early antigen (EBV-EA) activation on Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Nishizawa et al., 1989). Raji cell is a human cell line from hematopoietic origin (Fadeel, 2005). The chemical derivatives include **5**, **6**, **8**, **13**, **14**, **17**, **18**, **19**, and **20** showed more potent anti-cancer activities than the natural product **4** as demonstrated in **Table 11**. The *in vivo* anti-skin tumor promoting activity of the derivative compounds **5**, **14**, and **17** were undertaken by using mouse skin. The chemical structures of these compounds were identified as shown in **Figure 14**.

Table 11 Percentages of inhibitory effect on EBV-EA activation on Raji cell inducing with TPA of seventeen derivatives of 3-oxo-24-cycloarten-21-oic acid isolated from leaves of *L. domesticum* extract (Nishizawa et al., 1989).

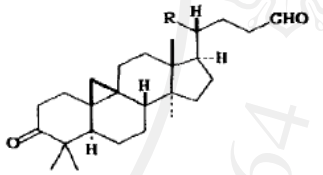
Compounds	Inhibitory effects (%)			
	32 nM	16 nM	3.2 nM	0.32 nM
4 (3-oxo-24-cycloarten-21-oic acid)	0%	30.5%	86.9%	100%
5	0%	0%	74.3%	95.7%
6	6.7%	43.7%	77.1%	92.1%
8	0%	20.5%	80.4%	100%
13	0%	0%	78%	100%
14	0%	0%	32.7%	71.4%
17	0%	0%	37.5%	92.7%
18	0%	0%	57.5%	100%
19	20.2%	70.1%	100%	100%
20	0%	35.5%	72.9%	90.1%



5; R^1, R^2 = O, R^3 = $COOCH_3$



14; R = $COOCH_3$



17; R = $COOCH_3$

Figure 14 Chemical derivatives of 3-oxo-24-cycloarten-21-oic acid isolated from leaves of *L. domesticum* extract which were further studied on *in vivo* anti-skin tumor in mouse skin (Nishizawa et al., 1989).

The anti-bacterial activities of *L. domesticum* were obtained from the separated bioactive compounds from its twig and bark. The bioactive compounds existing in the extract from twigs of *L. domesticum* was isolated and determined for *in vitro* anti-bacterial activities (Dong et al., 2011). This study reported that the nine compounds isolated from the twig extract including lamesticumin A, the ethanolysis product of lamesticumin A, lamesticumins B - F, lansic acid 3-ethyl ester, and ethyl lansiolate, exhibited anti-bacterial against *Staphylococcus aureus*, *Staphylococcus*

epidermidis, Micrococcus luteus, Bacillus subtilis, Micrococcus pyogenes and *Bacillus cereus* as shown in **Table 12**.

The methanolic extract of bark of *L. domesticum* has been partitionated and investigated on anti-bacterial activity (Mayanti et al., 2007). The ethyl acetate fractions exhibited anti-bacterial against *B. cereus* and *E. coli* with inhibitory zone 12.5 and 14 mm, respectively. The chemical compound, named 14-hydroxy-7-onoceradienedione which was separated from the fraction, showed the lower anti-bacterial activity than the ethyl acetate fraction.

Table 12 Anti-bacterial activities of nine compounds isolated from ethanolic extract from the twigs of *L. domesticum* (**Figure 9**) (Dong et al., 2011).

Bacteria	MIC (μg/ml)									
	Magnolol (positive control)	1	2	3	4	5	6	7	8	9
S. aureus	25	6.25	-	6.25	6.25	-	-	-	50	6.25
S. epidermis	12.5	12.5	12.5	12.5	12.5	-	-	-	12.5	12.5
M. luteus	12.5	6.25	50	3.12	6.25	-	-	-	-	6.25
B. subtilis	12.5	3.12	12.5	3.12	3.12	6.25	12.5	12.5	12.5	3.12
M. pyogenes	25	3.12	-	3.12	3.12	-	6.25	-	-	3.12
B. cereus	12.5	3.12	3.12	3.12	3.12	3.12	3.12	3.12	-	3.12

Note: MIC was defined as the lowest concentration that inhibited visible growth. "–" was defined to be inactive.

B. Cosmetics

The cosmetic properties of extracts from *L. domesticum* were observed both *in vitro* assay such as DPPH radical scavenging and tyrosinase inhibition activities, and *in vivo* assay such as skin moisture content and skin depigmentation which has been evaluated in human volunteers, were published. However, bioactive compounds of those natural extracts from *L. domesticum* have never been published. According to the responsibility for reliable safety profile, the

extract of *L. domesticum* containing in cosmetic formulations were tested on irritation and allergic in animals and human volunteers. The *in vitro* and *in vivo* cosmetic assessments of the extract from various parts of *L. domesticum* were listed in **Table 13**.

Table 13 The cosmetic properties of extracts from various parts of *L. domesticum*

Part used	Cosmetic properties	References
Fruit peel extracted with ethanol	*In vitro* - Free radical scavenging activity expressed as trolox equivalent antioxidant capacity (TEAC) with the value of 0.507 ± 0.002 mM/mg dry extract	(Tachakittirungrod et al., 2007)
Flesh fruit extracted with hydroethanol	*In vitro* - DPPH radical scavenging activity (data not shown) - Tyrosinase inhibition activity (data not shown)	(Shimada et al., 1992; Tilaar et al., 2007a; Vanni et al., 1990).
	In vivo - 2-5% of dry extract recommended for skin care application - No irritation of allergic skin observed in SCPT and ROPT in all volunteers at 1 and 3% of extract - 5% of extract cause irritation in 1.9% of all volunteers - 50 mg of the extract in lotion base showed no toxic HET-CAM test - Performed on a panel of 30 female volunteers aged 32-52 years old during 4 weeks - Skin moisture content was measured using Corneometer® CM 820 - Lightening effect was measured using a Mexameter® MX 16 - Significantly increased skin moisture content and decrease the skin melanin index (data not shown)	(Martha et al., 2007) (Serup et al., 1995; Tilaar et al., 2007a)

Note: SCPT represented for single closed patch test, ROPT represented for repeated open patch test and HET-CAM represented for Hen's egg testing of chorioallantoic membrane.

C. Foods and food supplements

The flesh of the *L. domesticum* is practically eaten fresh or served as a dessert (Morton, 1987). The edible portion of *L. domesticum* composed of

several kind of food values including carbohydrates, fiber, protein, phosphorus, carotene and others (**Table 14**).

Table 14 Food value per 100 g of edible portion of *L. domesticum* (Morton, 1987)

Food value	Content
Moisture	86.5 g
Protein	0.8 g
Carbohydrates	9.5 g
Fiber	2.3 g
Calcium	20.0 mg
Phosphorus	30.0 mg
Carotene (vitamin A)	13.0 I.U.
Thiamine	89 µg
Riboflavin	124 µg
Ascorbic Acid	1.0 mg

Note: 1 I.U. of vitamin A equals to 0.0006 mg of supplemental carotene.

D. Agriculture

Insecticide or anti-feedant activity of the chemical compounds containing in the bark extract of *L. domestiucum* have been studied. The fruit peel of *L. domesticum* is traditionally known to be toxic to domestic animals. The anti-feedant triterpenoid namely iso-onoceratriene which are 3-keto-22-hydroxyonoceradiene, onoceradienedione, lansiolic acid, lansiolic acid A and 3-keto lansiolic acid (Mayanti et al., 2007). An investigation has studied on the anti-feedant activity of six compounds isolated from fruit peel extract of *L. domesticum* (Omar et al., 2007). The isolated terpenes were tested for insect anti-feedant activity against rice weevil (*Sitophilus oryzae* L.) using a flour disk bioassay. Among the isolated six

derivatives, onoceratriene, 3-keto-22-hydroxyonoceradiene, onoceradienedione, lansiolic acid and lansiolic acid A, were shown to exhibit significant anti-feedant activity (**Figure 15**).

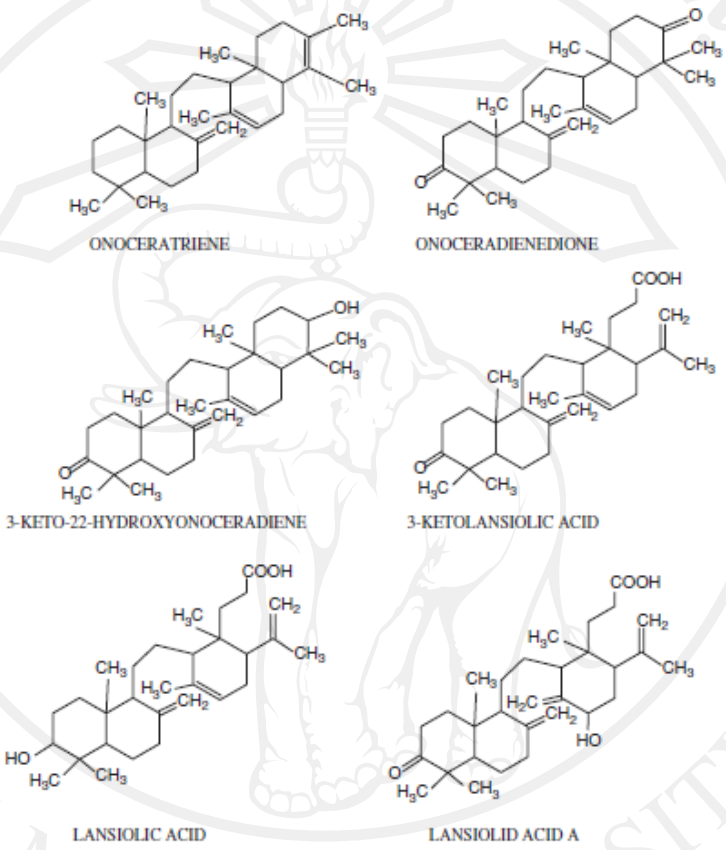


Figure 15 Structures of triterpenes isolated from *L. domesticum* which has insect antifeedant activity against rice weevil except 3-ketolansiolic acid (Omar et al., 2007)

1.4.4 The extraction processes of natural products

Plants contain a large variety of chemicals, therefore extracted solvents or procedures are importance in order to obtain more quantities of bioactive compounds. The first step of the extraction usually begins with randomized extraction in order to obtain crude extract and screening for biological activity of the

crude extract, before selection of the most active extract will be further development (Mccloud, 2010). Frequently, the impurities of the crude extract which composed of many complex chemicals decrease the biological activity of the active compounds. Hence, the crude extracts which have potential biological activity are regular purified as pure chemical substances or semi-purified fractions. However, nutraceuticals, cosmetics and fragrances not required for ultrapure products, which is the strong effect as the pharmaceutical and medical applications (Cannell, 1990).

1.4.4.1 Crude extracts

According to different polarities of the biomolecules existing in plants, the solubility of extracted solvent for crude extract preparation is very important. Crude extract containing various phytochemical constituents due to a broad polarities of solvents with solvating power of each extraction method including sonication and hot extraction, therefore, the crude extract may be impurity and shown low potent biological activity (Handa et al., 2008). The natural extracts that were prepared as crude extract include essential oil from various plants for cosmetic purposes were listed in **Table 15** (Krishnamurthy, 2001).

Table 15 Examples of the natural crude extracts and essential oil prepared from various plants for cosmetic purposes (Krishnamurthy, 2001)

Natural extract / essential oil	Scientific name	Cosmetic properties investigations
Alfalfa extract	*Medicago sativa* Linn.	Antioxidant
Aloe vera extract	*Aloe vera* Linn.	Wound healing, emollient and
Peanut extract	Arachis hypogaea Linn.	sunscreen
Artichoke extract	*Cyanara scolymus* Linn.	Emollient and anti-inflammatory
Blue elder berry extract	*Sambucus caerulea* Raf.	Antioxidant
Camellia oil	*Camellia sasanqua* Thunb.	Antioxidant
Chestnut rose extract	*Rosa roxburghii* Sweet.	Emollient
Phyllanthus extract	*Phyllanthus emblica* Linn.	Antioxidant
Rose hip oil	*Rosa moschata* Benth.	Antioxidant and anti-inflammatory
Rosemary extract	*Rosmarinus officinalis* Linn.	Emollient and antioxidant
Sea lettuce extract	*Ulva lactuca* Linn.	Antioxidant
		Anti-inflammatory and antioxidant

A. Ultrasound extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. This mechanism is an enhanced mass transfer and improved penetration of the solvent into the specimen due to the introduction of the ultrasound wave. However, the disadvantage of the procedure is the occasional deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents through formation of undesirable changes in the biomolecules. The ultrasonication is a suitable method for guava leaf extraction due to it yielded the extract with the significantly highest total phenolic content and antioxidant activities using free radical ABTS assay and ferric reducing antioxidant power (FRAP) assay (Nantitanon et al., 2010).

B. Hot extraction

In this process, the crude drug is boiled in organic solvents at their boiling temperatures in a specified volume solvent for a defined time; it is then cooled and strained or filtered. The soxhlet apparatus is a device employed for prevent the evaporation of organic solvents during hot extraction, but this device is not suitable for water extraction because of its high boiling temperature. The hot extraction is suitable for extracting heat-stable constituents in shorting time. The starting ratio of crude extract to solvent is fixed such as 1:4 or 1:16. The volume is then brought down to one-fourth of the original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and evaporated to obtain dried residue (Okorie et al., 1968; Sultana et al., 2009). The higher extract yields of various medicinal plants were obtained by the hot extraction technique with

higher amounts of total phenolic contents as well as better antioxidant activity than the extracts prepared using a shaker technique (Sultana et al., 2009).

C. Supercritical carbon dioxide fluid extraction ($scCO_2$)

Supercritical fluid extraction (SFE) is an alternative sample preparation method for reduced use of organic solvents and increased volume of crude extract. The factors to consider include temperature, pressure, sample volume, modifier (co-solvent) addition, flow and pressure control, and restrictors. The advantages of CO_2 used as the extracting fluid were its favorable physical properties, inexpensive, safe and abundant. However, the major deterrent in the commercial application of the extraction process is its prohibitive capital investment. The antioxidant activity of *Oryza sativa* extracted by using supercritical CO_2 fluid, according to high content of unsaturated fatty acid and total phenolic compounds (Ruksiriwanich et al., 2011).

1.4.4.2 Semi-purified extracts

Plant crude extracts usually contain large amounts of carbohydrates and/or lipoidal material and the concentration of the bioactive components in the crude extract may be low. To concentrate and obtain semi-purified extracts before analysis, some strategies including liquid-liquid partitioning, column chromatography and solid-lipid phase extraction based on polarity have been commonly used. In general, elimination of lipoidal material can be achieved by washing the crude extract with non-polar solvents. The semi-purified extract using liquid-liquid partition is widely applied as natural active ingredients in cosmetics. The application of the ethyl acetate fraction prepared by liquid-liquid partition from *Caulerpa racemosa* exhibited the strong antioxidant activity against DPPH free

radicals and also presented high phenolic contents (Li et al., 2012). The compartison of between advantages and limitations of solvent extraction, supercritical fluid extraction and ultrasonication methods were shown in **Table 16**.

Table 16 Advantages and disadvantages of the popular extraction methods for crude extract preparation from plants (Mercer et al., 2011)

Extraction method	Advantages	Limitations	References
Solvent extraction	Solvents used are relatively inexpensive which the results are reproducible	Most organic solvents are highly flammable or toxic, large volume of solvent is required	(Galloway et al., 2004; Herrero et al., 2004)
Supercritical fluid extraction	Non toxic (no organic solvent residue in extracts), non-flammable and simple operation	High power consumption, expensive and difficult to scale up	(Macı´as-Sa´Nchez et al., 2005; Pawliszyn, 1993)
Ultrasonication	Reduced extraction time, solvent consumption, improved release of cell contents into bulk medium	High power consumption, difficult to scale up	(Luque-Garcı´ et al., 2003; Martin, 1993)

1.4.4.3 Discolor subfractions from the semi-purified extracts by partition technique

The pigments existing in the plant extracts become an important problem for cosmetic formulations including chlorophyll, carotene and xanthophylls (Yoshimura et al., 2001). Chlorophyll often interferes with the analysis of bioactives from plants. Proper discoloration of plant extract is important in order to achieve the highest accuracy. In addition, color of pigments in plant extracts contribute to organoleptic appearance of cosmetics. The selective extraction method should reduce or remove chlorophyll without affecting the actual analytical compounds. The peaks of the chromatogram should be simplified to identification and interpretation. The chlorophyll removal from *Arabidopsis thaliana* extract using chloroform extraction has been revealed. The UV/Vis spectroscopy profile of the *A.*

thaliana extract indicated that chlorophyll was decreased after extracted with chloroform as shown in **Figure 16** (Mitra et al., 2009).

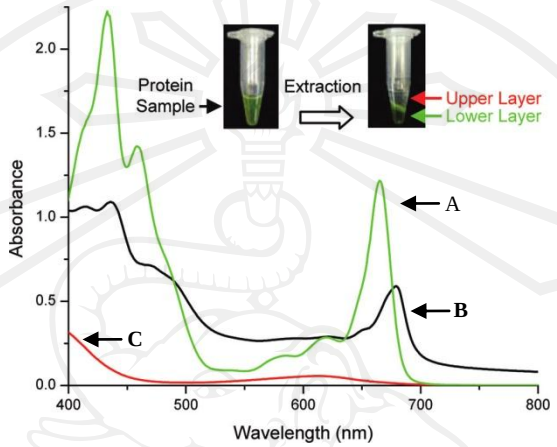


Figure 16 The UV/Vis spectroscopy data of *A. thaliana* extract removal chlorophyll using chloroform extraction. The spectrum of the extract before chlorophyll removal exhibited two maximum peak of chlorophyll (A), while the upper (B) and lower (C) layer which were aqueous phase and chloroform phase respectively showing the broad spectrum (Mitra et al., 2009).

1.4.5 Characteristic analysis of the extracts from the natural products

1.4.5.1 Phytochemical screening test

Phytochemical screening test is the quality investigation of bioactive compounds produced in plants which composed of a large number of secondary metabolites (**Table 17**). In general, the phytochemical test is verified according to the chemical reactions of the bioactive constituents containing in plant extracts and the adding chemical substances. Thin layer chromatography (TLC) with specific spraying reagent is also widely used for experiment. The final productions of

complete chemical reactions can be observed as positive results. Plant extracts are usually screened for phytochemical constituents including alkaloids, flavonoids, saponins, tannins, reducing sugars, steroids and triterpenoids (Harborne, 2005).

Table 17 Phytochemical screening tests of the plant extracts (Aslan et al., 2011; Kujur et al., 2010)

Phytochemical screening test	Used reagents	Positive results
Alkaloids	- Mayer's reagent	- yellow color precipitate
	- Drafendroff's reagent	- red precipitat
Anthraquinones	- Borntrager's test	- rose pink
Anthocyanins	- Sodium hydroxide test	- bluish green color
Carotenoids	- Sulfuric acid test	- Blue color
Flavonoids	- Shinoda test	- magenta color
Reducing sugar	- Benedict's ragent	- brick red precipitate
	- Fehling's reagent	-
Saponins	- Foam test	- 1 cm layer of bubbles appeared for 15 min
Stearoids	- Liberman Burchard's test	- brown ring
Tannins	- Ferric chloride test	- blue or green color
Terpenoids	- TLC spraying with vanillin-sulfuric acid	- deep purple spot

Alkaloids are nitrogen-containing compounds widely distributed in different plants. Alkaloids are normally grouped on the basic of their ring system, including indolizidine- and quinolizidine-based systems and quinoline-, quinazoline-, and acridone-based systems were recently reviewed (Michael, 2004) (**Figure 17**). 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. The alkaloid constituents of leaves of *Cassia fistula* L. and berries of *Hippophae rhamnoides* L. have been preliminary tested for alkaloids using Mayer's and Dragendorff's reagents. Alkaloids were found in *C. fistula* and *H. rhamnoides* extracts performed by using both reagents (Khan et al., 2012).

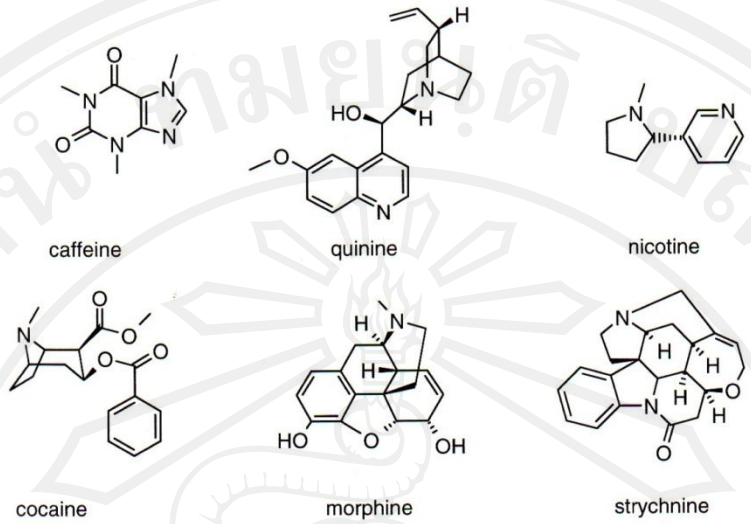


Figure 17 Chemical structures of some common alkaloids (Michael, 2004)

Flavonoids have two benzene rings separated by a propane unit. They are generally water-soluble compounds. The more conjugated compounds are often brightly colored. These include the chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavones (Williams et al., 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). The flavonoids were found in Sudanese medicinal plants extract performed by using HCl and magnesium ribbon which pink or magenta-red color were observed as positive results (El-Kamali et al., 2010).

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

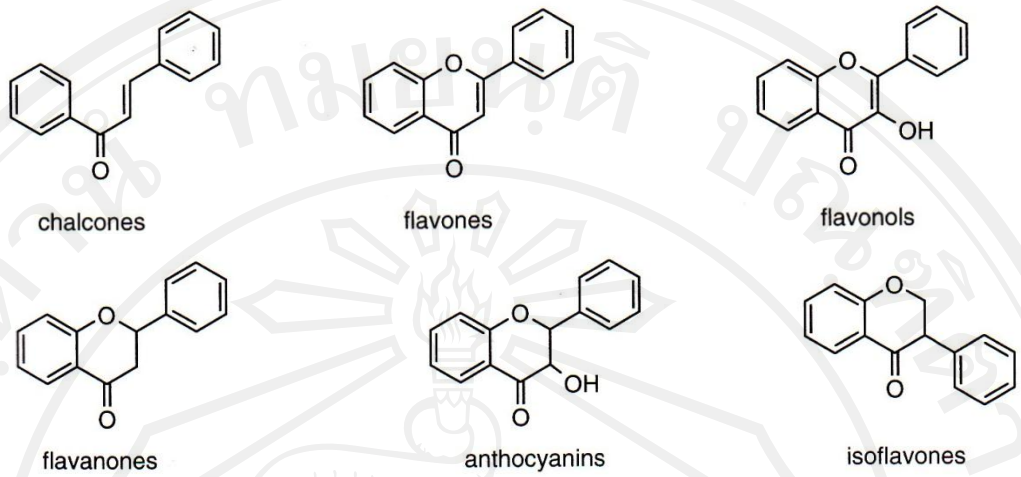


Figure 18 Chemical structures of flavonoid classes (Williams et al., 2004)

Soaproot (*Chlorogalum pomeridianum*), Soapbark (*Quillaja saponaria*) and Soapnut (*Sapindus mukurossi*) (Hostettman et al., 1995). Saponins existing in leaf extracts of *Vitex doniana* and *Mucuna pruriens* performed by vigorous shaking gave the durable of bubbles after 1 h have been studied (Onwukaeme et al., 2007). Glycyrrhizin is a major saponin containing in the root of *Glycyrrhiza glabra* L. (Licorice). Ginsinoside are a class of steroid glycosides and triterpene saponins which is found exclusively in the plant genus Panax (ginseng) (**Figure 19**).

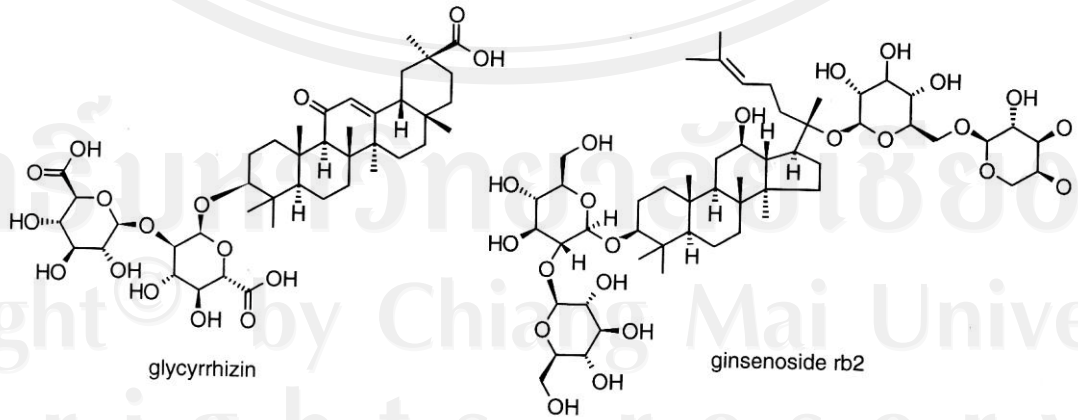
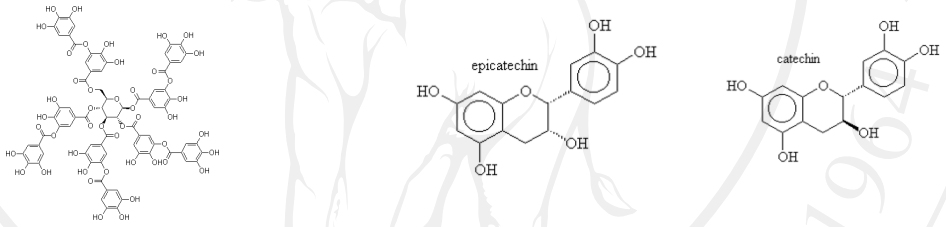


Figure 19 Chemical structures of saponins (Onwukaeme et al., 2007)

Tannins are water-soluble oligomers, rich in phenolic groups, capable of binding or precipitating water-soluble protein. 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 20**). Hydrolysable tannins are ester of a sugar with one or more trihydroxy benzene carboxylic acids (gallic acid). Tannins produce different colors with ferric chloride (either blue, blue black, or green to greenish black) according to the type of tannin (Hagerman et al., 1989). The leaves extract of *Moringa oleifera* containing tannins has been applied as pharmaceutical and medicinal plants (Kasolo et al., 2010).



Tannic acid
Condensed tannin

Hydrolysable tannin

Figure 20 Common condensed tannin and hydrolysable tannin in plants (Hagerman et al., 1989)

Reducing sugars is any sugar that has an aldehyde or a ketone group. 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. Benedict's reagent and Fehling's solution are used to test for the presence of a reducing sugar. The reducing sugar reduces copper 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 (Campbell et al., 2012). The reducing sugars including glucose, fructose and sucrose (**Figure 21**) were qualitatively observed in the extracts from *Mucuna pruriens* and *Mucuna bracteata*. The brick red precipitate presented after added Fehling's solution with heating (Kumar et al., 2009).

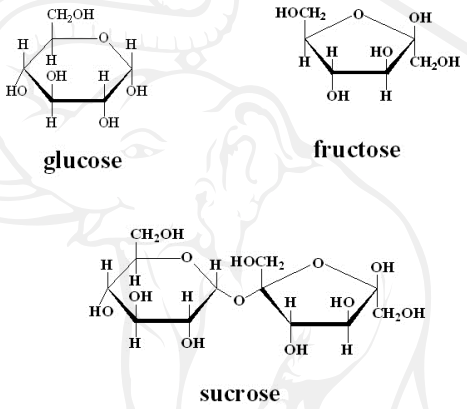


Figure 21 The chemical structure of the reducing sugars which are qualitatively determined in the plant extracts by Fehling's solution test (Campbell et al., 2012)

Anthocyanins are glycosylated polyhydroxy and polymethoxy derivates of 2-phenylbenzopyrylium (flavylium) salt which are water soluble pigments widely distributed in color of innumerable edible fruits. They appear to be red, purple, or blue depending on the pH. Anthocyanins belong to a class of molecules called flavonoids synthesized via the phenylpropanoid pathway; they are odorless and nearly flavorless, contributing to taste as a moderately astringent sensation. Anthocyanins occur in all tissues of higher plants, including leaves, stems, roots, flowers, and fruits. The most common anthocyanins present in colorful edible fruits such as pelargonidin 3-glycoside, cyanidin 3-glucoside and malvidin 3-glucoside. Sodium hydroxide test

is used for anthocyanins determination which blue color indicates the presence of anthocyanins. There are several reports concerning anthocyanins in purple corn plants. *Zea mays* L. is a rich source of anthocyanins (Jing et al., 2005). The chemical structures of these anthocyanins in the purple corn have investigated for functional food (Jing et al., 2005) (**Figure 22**).

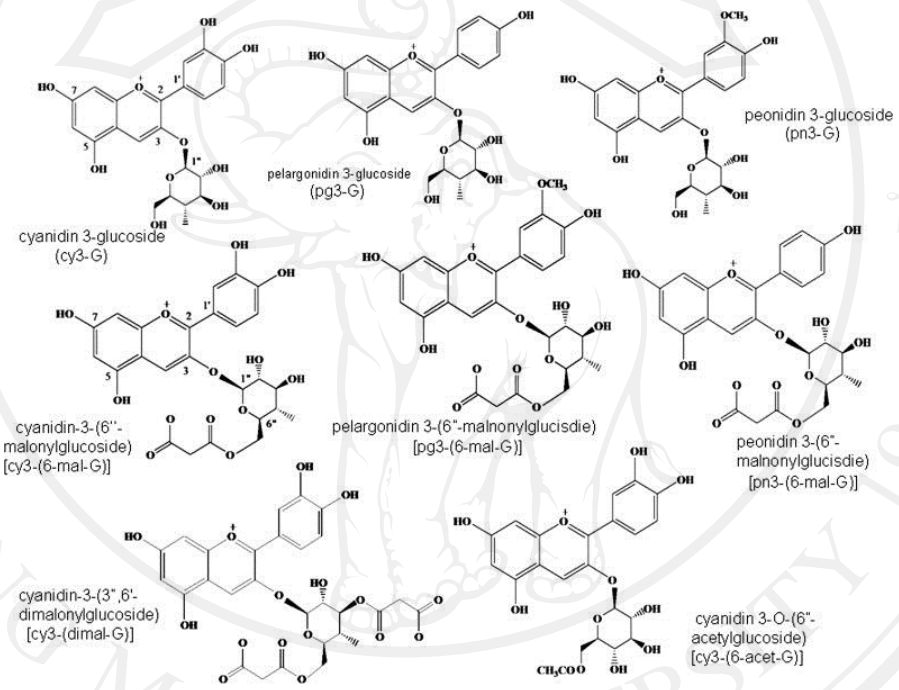
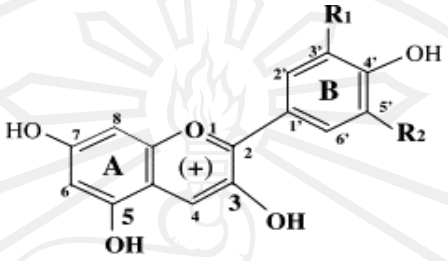


Figure 22 Major anthocyanins containing in the purple corn (Jing et al., 2005)

Anthraquinones are aromatic organic compound in plants. The keto groups are located on the central ring of its chemical skeleton. It is a yellow highly crystalline solid, poorly soluble in water, but soluble in hot organic solvents. Borntrager's test is used for the detection of anthraquinones. The free anthraquinones are detected with ammonia which the reaction produces a pink, red or violet color in the ammoniacal phase. Bound anthraquionones can cleavable by boiling with sulfuric

acid (Evans, 2002). Roots, stems, flowers and leaves of *Ceratotheca triloba* contained anthocyanins and its derivatives as major compounds (Mohanlall et al., 2011).



Aglycon	R_1	R_2	Wavelength (nm) / color
Pelargonidin	H	H	494 nm / orange
Cyanidin	OH	H	506 nm / orane-red
Peonidin	OMe	H	506 nm / orane-red
Delphinidin	OH	OH	508 nm / red
Petunidin	OMe	OH	508 nm / red
Malvidin	OMe	OMe	510 nm / bluish-red

Figure 23 Common anthocyanins visible in various color edible plants (Giusti et al., 2003)

Carotenoids are yellow, orange, and red pigments synthesized by plants. The most common carotenoids in plants are α-carotene, β-carotene, β -cryptoxanthin, lutein, zeaxanthin, and lycopeneA. The distinctive characteristic is an extensive conjugated double-bond system, which serves as the light-absorbing chromophore responsible for the color of plants. In nature, they exist primarily in the more stable all-*trans* isomeric form, but *cis* isomers presence in small amounts. The determination of carotenoids is carried out by sulfuric acid test, the presence of blue color indicates to carotenoids. Carotenoids exist in the root skeel extract of *Mondia whytei* (hook.f) detecting using sulfuric acid test have been reported (Githinji et al., 2012). The chemical structures of carotenoids were shown in **Figure 24**.

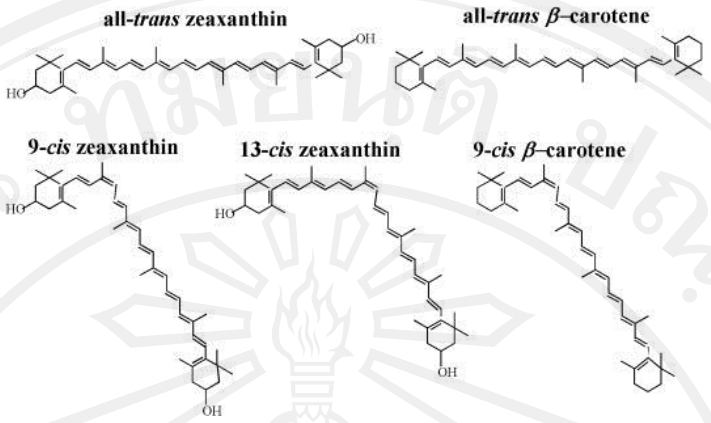


Figure 24 Chemical structure of carotenoids presence in all-*trans* and *cis* isomers of zeaxanthin and β-carotene (Widomska et al., 2009)

Steroids are a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other. The core of steroids is composed of twenty carbon atoms bonded together that take the form of four fused rings which are three cyclohexane rings and one cyclopentane ring. The steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings. Hundreds of distinct steroids are found in plants, animals, and fungi. Liberman-Berchard's test is used for detection of steroids. After adding acetic anhydride, the brown ring formed on the top of solution indicated presence steroids. Steroids presented in the leaves, stem and root of *Plectranthus glandulosis* extracted with hexane and ethyl actate (Egwaikhide et al., 2007). There are many common steroids found in plant and animals (**Figure 25**).

Triterpenoids are groups of terpenoid derivatives containing 30 carbon atoms which present in the plant kingdom. Triterpenoids divided to two main groups which are the tetracyclic triterpenoids and the pentacyclic triterpenoids. There are several

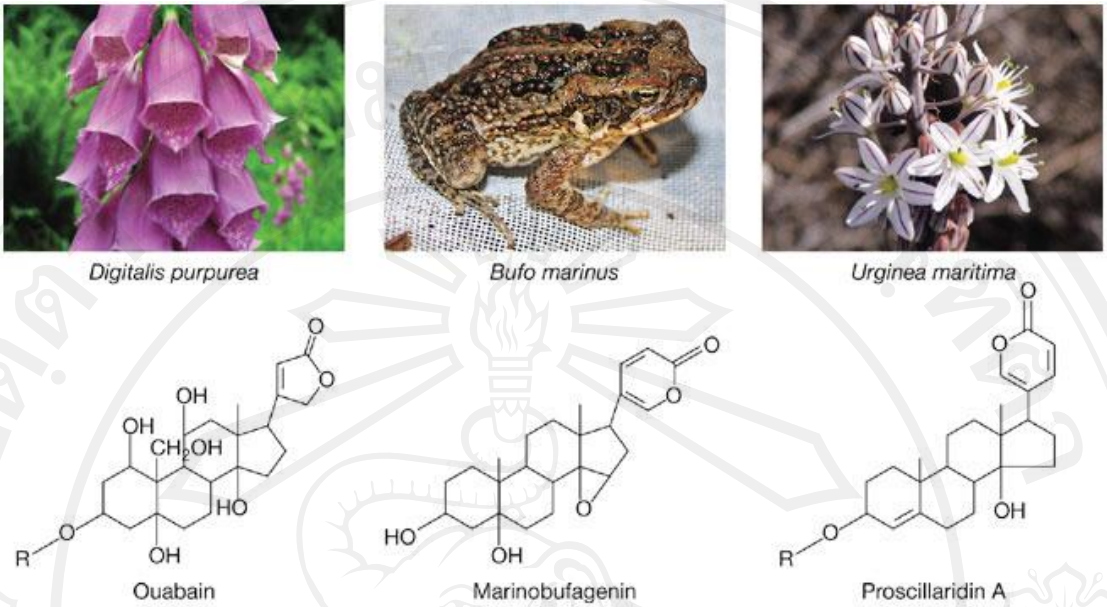
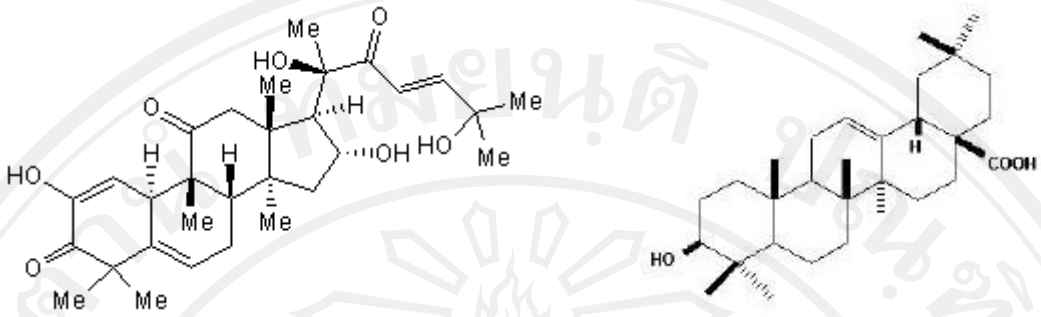


Figure 25 Major steroids determined in plants such as ouabain from *Digitalis purpurea* and Proscillararidin A from *Urginea maritime*, and in animals such as marinobufagenin from *Bufo marinus* (Bagrov et al., 2008)

triterpenoids including squalene derivatives, lanostanes, cycloartanes, dammaranes, euphanes, tetranortriterpenoids, quassinoids, lupanes, oleananes, friedelanes, ursanes, hopanes, fernanes, sipholanes, isomalabaricanes and serratanes, have been studied on chemical structure and biological activities (Guangyi et al., 2005; Sam et al., 2008). Triterpenoids screeing test is determined by TLC development and spraying with vanillin solution, the deep purple spot indicates the presence of triterpenoids. Meliacinolactol, limocin C and limocin D are further constituents of *Azadirachta indica* (Neem tree) which posses antioxidant activity (Siddiqui et al., 2001). The example of tetracyclic and pentacyclic triterpenoids were shown in **Figure 26**.



Cucurbitacins
(tetracyclic triterpenoid)

Oleanolic acid
(Pentacyclic triterpenoid)

Figure 26 Example of tetracyclic and pentacyclic triterpenoids (Vikram et al., 2010)

1.4.5.2 Specification test

Specification test is used as a part of quality control strategy designed to ensure efficacy and safety. Specification is a characterization of specific information of each substance. The appropriate physical and chemical specification test of each ingredient including botanical extract, raw material and finish product, are defined. The physical specification test is applied for physical appearance investigations including preference solubility, pH, odor, color, etc. The chemical specification tests are discriminant analytical techniques such as HPLC, GC/MS and NMR which limit chemical purities and probably toxic contamination like heavy metal. The microbiological specifications are essential for ingredients of biological origin such as these derived from plants, animals or biotechnology and finish products which occasionally composed of growth factors influential to microbiology. The standardization of the leaves extract from *Ginkgo biloba* as medicinal applications is listed in **Table 1**8 (Beek, 2002).

Solubility test is a procedure based on attempting to dissolve chemicals in various solvents with increasingly rigorous mechanical techniques in the order of

Table 18 Examples of specification for standardized Ginko extract (Beek, 2002)

Description	Brown powder with characteristic smell
Identity	Green-brown color after adding FeCl3 to a 0.1% solution (g/v) in alcohol-water (1:1)
Heavy metals	Not more than 20 ppm
Arsenic	Not more than 2 ppm
Ginkgolic acid	Not more than 10 ppm
Loss on drying	Not more than 5.0% (80ºC, vacuum)
Residue on ignition	Not more than 1.0%
Total flavonoid content	Not less than 24.0% (HPLC-UV)
Total terpene trilactone content	Not less than 6.0% (HPLC-RI)

preference at relatively concentrations. If the chemical does not dissolve, the volume of solvent is increased and the sequence of mechanical procedures is repeated at the lower concentrations. A solubility study of excipient is essential to develop a stable cosmetic dosage form, especially when the active agent is unstable (Lopes et al., 2011). The relative stability of carotenoids. This study revealed that the solubility of β-carotene which is a non-polar carotenoids, in triglycerides was significantly higher than zeaxanthin which is a polar carotenoids. The result indicated that the solubility of both carotenoids increased when the chain-length of the triglycerides' fatty acids decreased (Borel et al., 1996).

Chemical stability test is a characterization based on the reaction of the test sample and chemical solution. The chemical characteristic of the plant extracts influential to the selection of ingredients which are applied in the formulations. The chemical stability is affected by several factors such as pH, chemical structure, metallic ions, and the presence of enzymes (Casta˜Neda-Ovando et al., 2009). The test is performed by using various chemical solution including acid, base, oxidizing agent and reducing agent, which effect to those factor. The high potent chemical

stability of the heartwood extract of *Artocarpus lakoocha* Roxb. has been developed as cosmetic active ingredient (Tengamnuay et al., 2006).

1.4.5.3 HPLC fingerprint profile

Fingerprinting technique has been widely accepted as a useful method for the evaluation and quality control of natural extracts and their finished products. High performance liquid chromatography (HPLC) fingerprint analysis has been regarded as one of the powerful analytical tools with least limitations as almost all types of samples can be analysed. The chromatographic fingerprint shows the result of similarity calculated based on the relative value of retention time with the selected marker compound as reference standard (Sirikatitham et al., 2007). Markers are chemically defined constituents in the extract or finished product for determination and identification. The HPLC fingerprint of the tablet containing the root extract of *Valeriana officinalis* by using valerenic acid and acetoxyvalerenic acid as marker compounds was shown in **Figure 28** (Lazarowych et al., 1998).

1.4.6 Topical pharmaceutical and cosmetic formulation development

1.4.6.1 Types of formulations

In pharmaceuticals and cosmetics, the topical formulation is defined as a vehicle of drug or active components which are applied for skin treatment and prevention of skin degeneration process (Draelos, 2001). For the physical characteristics of common formulations such as spreadability, rheological, adhesion and extrusion, were categorized in dosage forms include solutions (e.g., serum), collodion, suspensions, emulsions (e.g., lotions), semisolids (e.g., foams, ointments,

pastes, creams, and gels), solids (e.g., powders and aerosols), and sprays (Ueda et al., 2009).

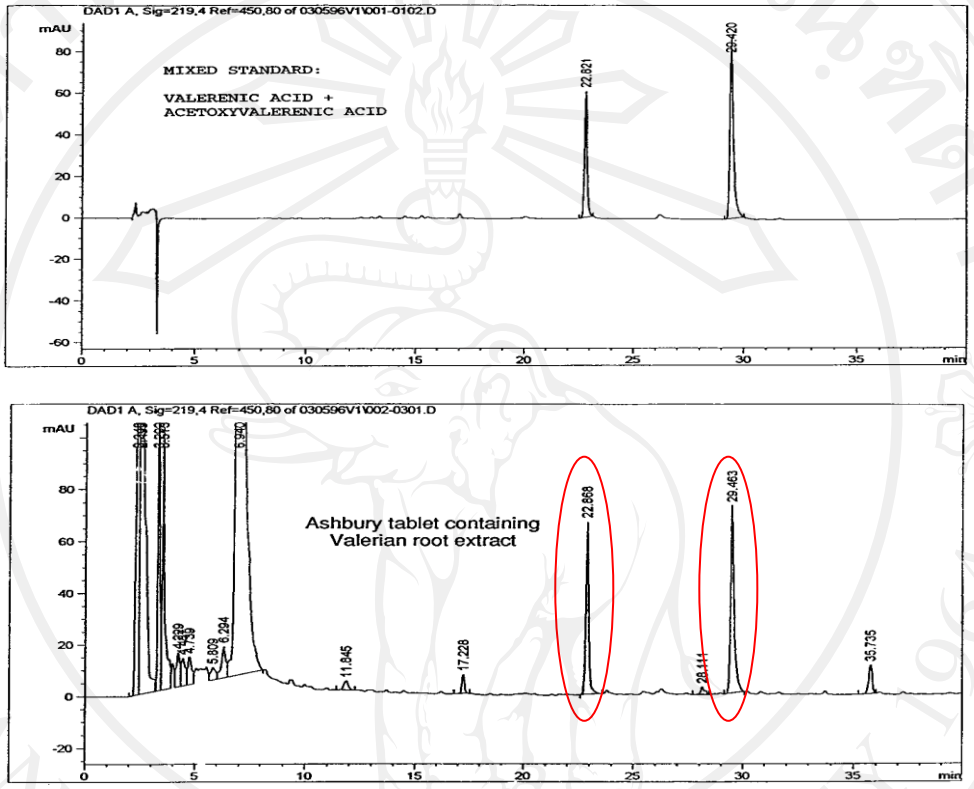


Figure 27 HPLC chromatograms of mixture of the marker compounds valerenic acid and acetoxyvalerenic acid (upper) and valerian tablets (lower) (Lazarowych et al., 1998)

Cream is a semisolid dosage form of the topical preparation. They are mixtures of oil and water which are divided into two types including oil-in-water (O/W) creams and water-in-oil (W/O) creams. Oil-in-water creams are more comfortable and cosmetically acceptable, because of less greasy and more easily washed off using water, than water-in-oil creams. However, water-in-oil creams are

more moisturising as they provide an oily barrier which reduces water loss from the skin.

Gel is a semi-solid, transparent or translucent which is stabilized or set by a three-dimensional lattice system. Gel can distinguish owing to phase system in two types include single phase and two phase gels. Single phase gels consist of organic macromolecules uniformly distributed throughout a liquid with no apparent boundary between the dispersed macromolecule and liquid, while the gel mass of two phase gel consists of a network of small discrete particles. Gels have advantages of an aesthetic standpoint as well as on application (Wittern, 1991).

Serum is defined as a uniformity liquid containing the biologically active substances dissolve in a mixture of diluents. Basically, serum is prepared without heating which avoid to degradation of the bioactive compounds and heated labile diluents. Water, oil or silicone based are used as prefer solvent of bioactive compounds. The physical appearance of serum including transparent or translucent, low viscosity and not adhesive to skin, become a dosage form that widely used in cosmetic products. The FDA does not regulate this type of cosmetic formulation, since the serum has not been proven by scientific trials and the company's claims may be exaggerated (Dahms et al., 1995).

1.4.6.2 Formulation development (Newman et al., 2002)

The cosmetic development process involves a number of activities which are carried out simultaneously, as shown by the simplified depiction in **Figure 28**. Once a molecule is discovered that has desirable biological activity. In this process, the method for identify its therapeutic doses are sought. Secondly, the preformulation study is performed in order to select the appropriate base formulation

for the pharmaceutical or cosmetic active ingredient which depended on the physico-chemical stability of the formulations. An interaction between active ingredient and base formulation was demonstrated and acceptable formulation component levels and the active components ranges were identified. Fourth, efficacy evaluation of the active molecule which depends on the nature of the drug substance and the final formulation are studied. These methods for efficacy assessment need to be evaluated relative to the intended formulations (Gaspar et al., 2008; Manosroi et al., 2002).

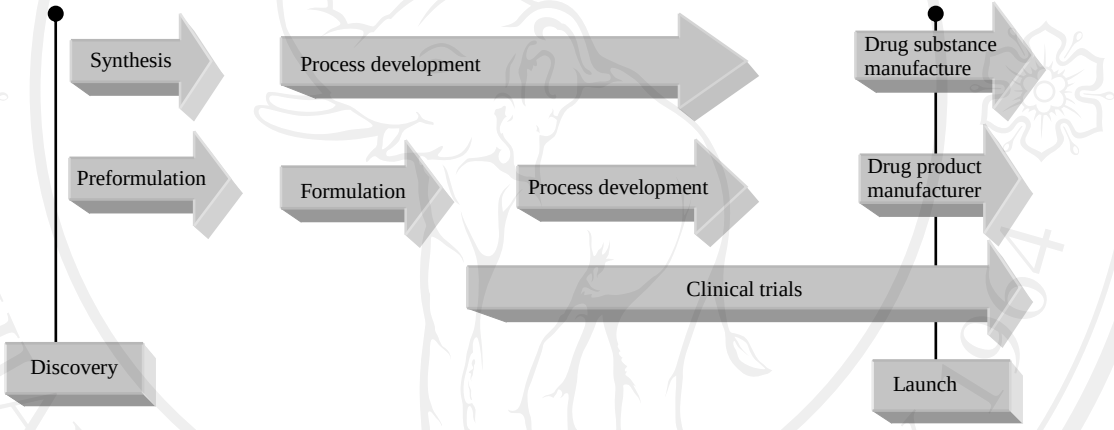


Figure 28 The process of pharmaceutical and cosmetic formulation development (Newman et al., 2002)

A. Chemical stability (Giral, 1947)

The chemical stability directly predicts the duration time of the pharmaceutical or cosmetic formulation degradation. The chemical stability is related to the bioactive compound decomposition which is frequently dependent on the nature of the base used in the formulation. The ingredient incompatibility is the most importance since it leads to decomposition of bioactive compounds. There are various chemical reactions that cause the incompatibility of the formulations including hydrolysis and oxidation. The duration time of the

bioactive degradation known as shelf-life can predict from the degradation rate constant of the chemical kinetic of these reactions. The degradation rate constant is estimated from the slope of the plot between bioactive concentrations versus times, by the least-squares fitting of various kinetic equations including zero order, first-order and second-order. The shelf life (t_{90}) and half life (t_{50}) are the time required when the bioactive compound in the formulations remained at 90 and 50% (Florence et al., 2006). The analysis of chemical stability can be monitored depending on development timelines and material availability. The preparations are determined for the remaining bioactive compound which is periodically analyzed by the appropriate methods. The simple analysis methods applied for chemical stability test of pharmaceutical and cosmetic preparations are HPLC, TLC and UV/Vis spectrophotometer.

B. Physical stability (Garrett, 1962)

The pharmaceutical and cosmetic products are susceptible to chemical decomposition when formulated in various dosage forms. The degradation not only leads to a loss of potency of the drug, but also causes changes in the physical appearance of the dosage forms, for example, discoloration and phase separation. Physical stabilities are usually determined under accelerated conditions. The preparations should be evaluated for clarity, homogeneity, pH, resuspendability (for lotions), consistency, viscosity, and particle size distribution (for suspensions, when feasible). Evaluation of eye care products (e.g., creams, ointments, solutions, and suspensions) should include the following additional attributes which are particulate matter and extractable. The determination of the hygroscopicity and solubility may be required for new active ingredients for the specification purpose.

1.4.7 *In vitro* anti-aging assays

1.4.7.1 DPPH radical scavenging 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 (Letelier et al., 2008; Suja et al., 2005). 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. There are many reports studied on the DPPH radical scavenging activity of Thai medicinal plant. Thai Lanna medicinal plants including *Terminalia chebula* has high antioxidant activity against DPPH radical scavenging activity (Manosroi et al., 2010).

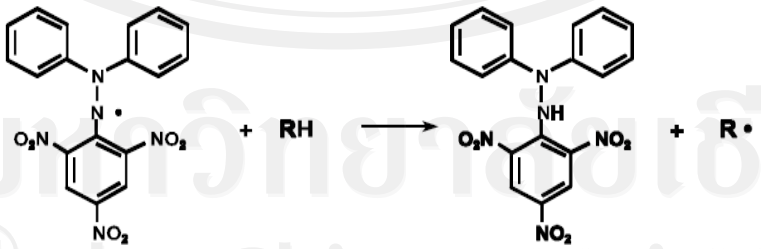


Figure 29 Reaction of the DPPH radical in the presence of the antioxidant during the DPPH assay (Prakash, 2001)

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 et al., 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). *Mentha longifolia* L. containing polyphenol compounds has strong potential metal chelating activity (Rice-Evans et al., 1996).

1.4.7.3 Lipid peroxidation inhibition by ferric-thiocyanate complex activity assay

The available methods to monitor lipid oxidation can be classified into five groups including the absorption of oxygen, the loss of initial substrates, the formation of free radicals, and the formation of primary and secondary oxidation products. Other chemical methods based on the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in an acidic medium and the formation of iron complexes have also been widely accepted. These methods spectrophotometrically measure the ability of lipid hydroperoxides to oxidize ferrous ions to ferric ions, which are complexed by either thiocyanate or xylenol orange (Eymard et al., 2003; Jiang et al., 1991). Ferric thiocyanate is a red-violet complex that shows strong absorption at 500– 510 nm (Dobarganes et al., 2002). The method of determining peroxide value by colorimetric detection of ferric thiocyanate is simple, reproducible, and more sensitive than the standard iodometric assay, and has been used to measure lipid

oxidation in milk products, fats, oils, and liposomes (Dobarganes et al., 2002; Eymard et al., 2003). The thiocyanate ions (SCN−) are added to make the presence of these ions in solution visible. These react with the Fe^{3+} ions to form a blood-red coloured complex as the following chemical reaction;

$$Fe^{3+}_{(aq)} + SCN^{-}_{(aq)} \rightarrow [FeSCN]^{2+}_{(aq)}$$

By comparing the intensity of the color of this solution with the colors of a series of standard solutions, with known Fe^{3+} concentrations, the concentration of iron in the sample can be determined. Lipid peroxidation inhibition activity of the 12 medicinal plants has been reported. The results from this study revealed that among all plants, *Cudrania tricuspidata*, *Zanthoxylum piperitum*, *Houttuynia cordata* and *Ulmus parvifolia*, showed effective inhibition on lipid peroxidation (Cho et al., 2003).

1.4.7.4 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 30**). 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. The compound derived from coumarin-resveratrol hybridization named 3-(3',4',5'-trihydroxyphenyl)-6,8-dihydroxycoumarin has high potent tyrosinase inhibition activity (0.27 mM) which was more than umbelliferone (0.42 mM) that used as reference compound (Fais et al., 2009).

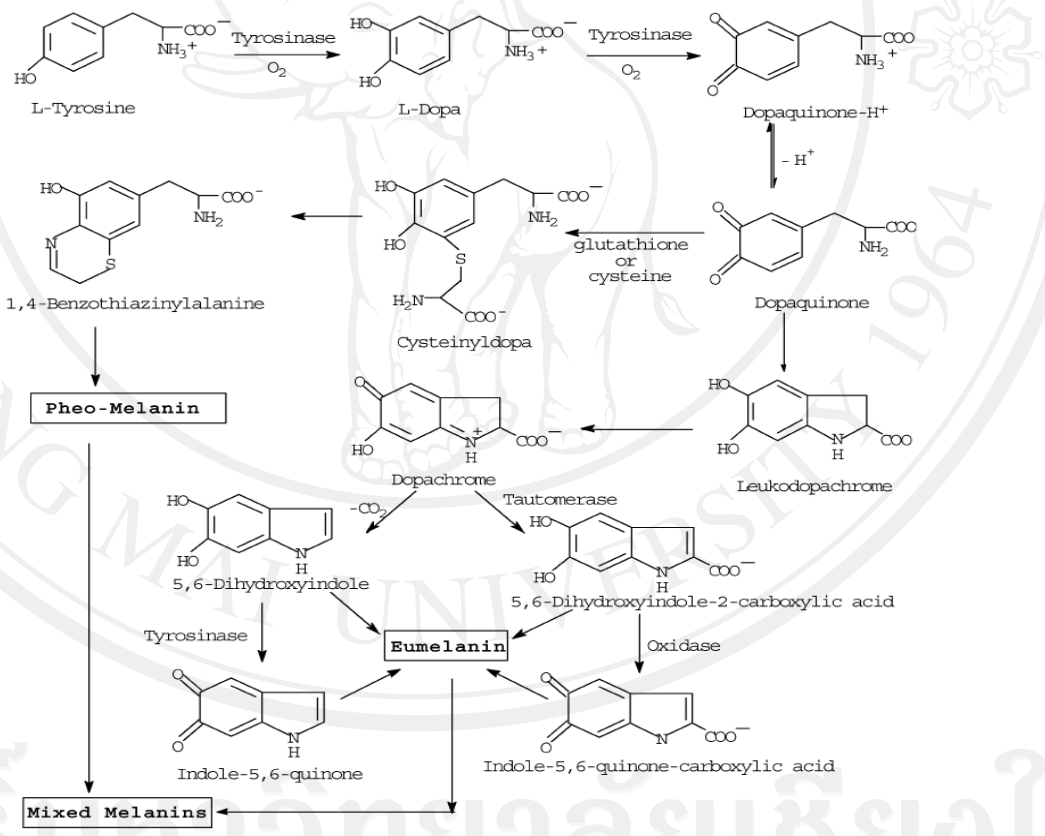


Figure 30 Melanogenesis pathway (Sung-Yum et al., 2003)

1.4.7.5 Gelatinolytic activity on MMP-2 inhibition (Zymography) in human skin fibroblasts

A. Types of collagen in the skin

Collagen is a major structural protein in the skin which gives the skin strength and durability and responses for the smooth, plump appearance of healthy young skin (Kim et al., 2007). Collagen is created by fibroblasts, which are specialized skin cells located in the dermis. When the receptors of fibroblast are bound to the signal molecules called fibroblast growth factors, the three protein chains wound together in a tight triple helix. When the receptors of fibroblast are bound to the signal molecules called fibroblast growth factors, the fibroblast begins the production of collagen (Patterson et al., 2001). The skin contains multiple types of collagen (**Table 19**), but the most significant collagen types are type I and type III collagen. Young skin is made up of fast-growing tissues, and has an abundance of type III collagen, which makes the skin soft and thick. In adult skin, type I collagen increases, when type III declines. Type I naturally regenerates itself until the skin reaches the peak of skin mechanical strength. Other types of collagen in the skin are V, VI, and XII which are found in much smaller amounts and appear to have a supportive role (Michel et al., 1991). There are several studies revealed that the bioactive constituents which are asiaticoside and madecassoside from the leaves extract of *Centella asiatica* exhibited the stimulation of type-I and type-III collagens, respectively (Bonte et al., 1995).

B. Gelatinase A (MMP-2) in aging skin

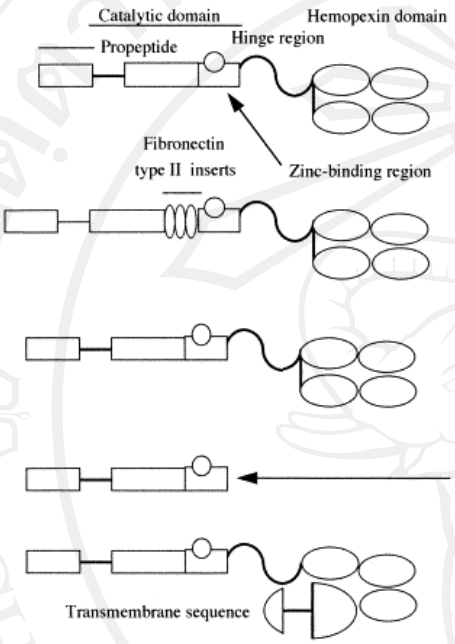
The MMPs are endopeptidases which compose of 21 human MMPs and the homologues from other species that can cleave virtually any component of the extracellular matrix (ECM). MMPs were divided into collagenases, gelatinases, stromelysins and matrilysins on the basis of their specificity for ECM

Table 19 Collagen family in the skin and other organs (Michel et al., 1991)

Types of collagen	Function
Type I	This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, cornea, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth.
Type II	Hyaline cartilage which makes up 50% of all cartilage protein. Vitreous humor of the eye
Type III	Collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus
Type IV	Basal lamina; eye lens. Also serves as part of the filtration system in capillaries and the glomeruli of nephron in the kidney.
Type V	Most interstitial tissue, assoc. with type I, associated with placenta
Type VI	Generally found alongside type I
Type VII	Epithelia (lining of GI tract, urinary tract, etc.) and orms anchoring fibrils in dermoepidermal junctions
Type VIII	Lining of blood vessels, Some endothelial cells
Type XII	Transmembrane collagen which interacts with integrin, fibronectin and components of basement membranes like nidogen and perlecan.

components and the common names of the MMPs reflect this classification as shown in the **Figure 31** (Michel et al., 1991). Gelatinases, previously named collagenases IV and now known as MMP-2 and MMP-9 of 72 and 92 Kda, respectively. They are secreted in a latent inactive form, and their conversion to the active species can be accomplished by other proteolytic enzymes, including other MMPs (Philips, 2010). Gelatin zymography is extremely sensitive for gelatinases like MMP-2 and MMP-9. MMP-2 and MMP-9 play a role in human skin aging and tumor development as well as in other cutaneous lesions are psoriasis and dermatitis (Patterson et al., 2001). The atrophy of collagen and elastin fibers in skin aging is predominantly from the

increased expression of their degradative enzymes, collagenases (MMP-1), gelatinases (MMP-2 and -9), and elastases (Wan et al., 2008).



Enzyme	Names	Latent/Active Mw (KDa)	Substrates
MMP-1	Interstitial collagenase	55/45	COL-I, II, III, IV, V,
MMP-8	Neutrophil collagenase	75/58	VII, VIII, X, XI,
MMP-13	Collagenase-3	60/48	FN, GL, PLP, PGs, TN,
MMP-18	Xenopus collagenase	55/?	pro-MMP-2, -9
MMP-2	Gelatinase-A 72kDa gelatinase	72/66	GL, COL-I, IV, V, VII, X, XI, XIV, EL, FN, LN, PLP, PGs, VN,
MMP-9	Gelatinase-B 92kDa gelatinase	92/86	pro-MMP-9, -13
MMP-3	Stromelysin-1	57/45	COL-II, III, IV, V, IX, X, XI, EL,
MMP-10	Stromelysin-2	57/44	FN, GL, LN, PLP, PGs, TN, VN,
MMP-11	Stromelysin-3	51/44	pro-MMP-1, -7, -8, -9, -13
MMP-19		?/?	
MMP-20	Enamelysin	?/?	Amelogenin
MMP-12	Metalloelastase	54/45/22	EL, COL-IV, GL, FN, VN, LN, PG
MMP-7	Matrilysin	28/19	COL-IV, X, EL, FN, GL, LN, PGs, PLP, TN, VN, pro-MMP-1, -2, -9
MMP-14	MT1-MMP	66/56	pro-MMP-2, -13,
MMP-15	MT2-MMP	72/?	COL-I, II, III, EL, FN, GL, LN,
MMP-16	MT3-MMP	64/52	PGs, TN, VN
MMP-17	MT4-MMP	?/?	

Figure 31 Type of MMPs divided by substrate and catalytic domain (Thomas et al., 1999)

C. MMP inhibitors for anti-aging skin

Currently, analytical methods for the determination of MMPs in biological samples include ELISA (enzyme-linked immunosorbent assays, including multiplexed ELISAs in the form of antibody arrays); zymography; optical methods such as near-IR (infrared) optical imaging, fluorescence, and surface plasmon resonance spectroscopy; the use of active-site probes followed by enzymatic digestion of the captured MMPs, and LC-MS/MS (liquid chromatography-mass spectroscopy) analysis of the digested MMPs (Strongin et al., 1995). Many studies have been identified the synthetic substance that are effective MMP inhibitors such as

N-biphenyl sulfonyl-phenylalanine hydroxamic acid (BPHA), pyrimidine-2,4,6,-triones (Ro 28-2653), batimastat, prinomastat (Ag 3340) and BAY 12-9566, but the synthesis of these substances are complicated (Gram et al., 2001). Natural products that have been identified to inhibit MMPs and elastase and simultaneously stimulate collagen and elastin such as *Polypodium leucotomos* extract, lutein, and xanthohumol. These products consist of polyphenols, carotenoids, or flavonoids with antioxidant, anti-inflammatory, photoprotective, or anticarcinogenic properties. *P. leucotomos* is rich in polyphenols which directly inhibits activities of MMPs and inhibits expression of MMPs in epidermal keratinocytes and fibroblasts and stimulates fibrillar collagens, elastin, and TGF-β in dermal fibroblasts (Philips et al., 2009; Philips et al., 2003). Lutein is a non-provitamin A carotenoid that inhibits epidermal hyperproliferation, expansion of mutated keratinocytes, and the infiltration of mast cells in response to solar radiation, and thereby photoaging (Astner et al., 2007). The mechanism to lutein's anti-aging and anti-photoaging effects includes the inhibition of MMP-to-TIMP ratio in dermal fibroblasts and the inhibition of cell loss and membrane damage in ultraviolet radiation-exposed fibroblasts (Philips et al., 2007). Xanthohumol, a flavonoid, directly inhibits MMPs (-1, -3, and -9) and elastase activities while dramatically increasing the expression of types I, III, and V collagens, elastin, fibrillin-1, and fibrillin-2 in dermal fibroblasts (Philips et al., 2010).

D. Zymography assay

Zymography method is widely used and identifies MMPs by the degradation of their preferential substrate and by their molecular weight (Beurden et al., 2005). All types of zymography are similar to gelatin zymography, the substrate simply differs depending on the MMP being analyzed. In zymography,

the proteins are separated by electrophoresis under denaturing (in the presence of SDS) and in nonreducing conditions; the separation is done in a polyacrylamide gel that contains the specific substrate. After electrophoresis, the gel is washed to remove SDS, following which the MMPs partially renature and recover their activity. The gel is then stained blue, and the MMPs are visible as clear bands against the blue substrate in the background and can be measured by densitometry. Proenzymes and active forms of MMPs can be distinguished by molecular weight (**Figure 32**).

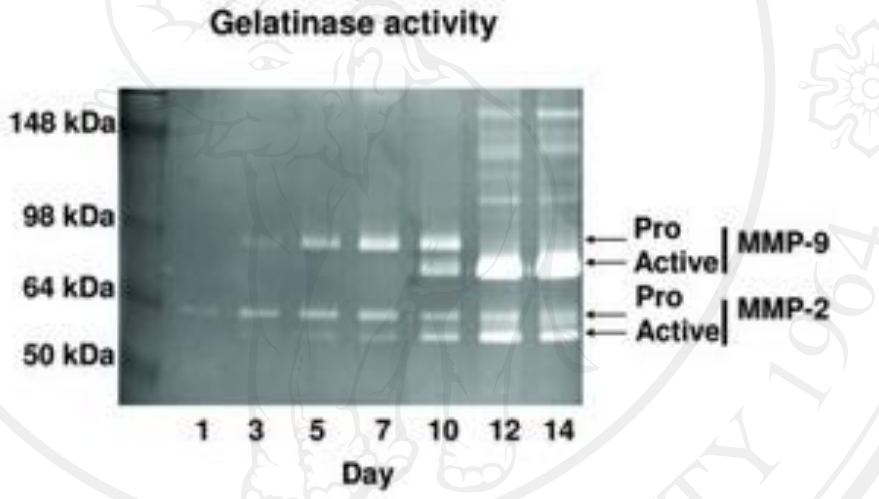


Figure 32 Zymograms of MMP-2 and -9 which exhibited distinguish molecular weight of pro- and active- enzymes (Milner et al., 2006)

Collagen zymography is also used for detection of MMP-1, -2, -9, and -13. *In situ* zymography, which can detect only active MMPs, allows the localization of MMPs in tissue sections and uses a substrate that is deposited on or under a frozen section of an unfixed tissue sample. It is important to use control slides with appropriate MMP inhibitors, to discriminate between the different classes of MMPs. The catalytic action of MMP enzymes is dependent on the zinc ion in the active site

and the enzymes are stabilized by calcium ions. Each MMP has a distinct protein substrate profile with partially overlapping profiles. With the zymographic method, sample proteins will be the first separated electrophoretically according to their specific molecular weight (MW) and electrical charge. After electrophoresis, the gels are renatured and the degradation of the substrate being studied is visualized by incubating the gels in standardized buffer with Ca^{+} and Zn^{+} ions added (Lopez-Avila et al., 2008).

1.4.7.6 Cytotoxicity by Sulphorhodamine B (SRB) assay in human skin fibroblasts

The cytotoxicity meaning covers both cytostatic and cytocidal effects. The SRB assay is sensitive, simple, reproducible and more rapid than the formazan-based assays and gives better linearity, a good signal-to-noise ratio and has a stable end-point that does not require a time-sensitive measurement, as the MTT or XTT assays (Fricker et al., 1996; Keepers et al., 1991). The sulphorhodamine B (SRB) assay is relies on the uptake of the negatively charged pink aminoxanthine dye, sulphorhodamine B by basic amino acids in the cells. The dye 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. The greater the number of cells, the greater amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense color and greater absorbance (Skehan et al., 1990). 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. With TCA fixed cultures, SRB gives a higher optical density (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).

1.4.8 *In vivo* anti-aging biological assays

In cosmetic industry, evaluation of efficacy and irritancy potential to human skin of any active compounds or formulations is a necessity. This must be done by means of *in vivo* and *in vitro* tests to determine the risk of irritation due to the contact between these compounds and human skin. Primary skin irritation is the production of reversible inflammatory changes in the skin following the application of a test substance as it involves the interaction of chemicals with the sensory receptors in the skin at the site of application.

1.4.8.1 Irritation test in rabbit

The most commonly used test is the rabbit skin irritation test described in the Organization for Economic Co-operation and Development (OECD) test guideline 404 and in the European Chemicals Bureau Annex V part B.4 which was initially described by Draize et al (Draize et al., 1944). The Draize test is an acute toxicity test devised in 1944 by Food and Drug Administration (FDA) toxicologists, John H. Draize and Jacob M. Spines.

Initially used for testing cosmetics, the procedure involves applying of a test substance to the skin of a restrained, conscious animal, and then leaving it for set

amount of time before rinsing it out and recording its effects. The animals are observed for signs of erythema and edema in the skin test. The test subject is commonly an albino rabbit, though other species are used too, including dogs. The animals are euthanized after testing if the test renders irreversible damage to the skin. Animals may be reused for testing purposes if the product tested causes no permanent damage. Animals are typically reused after a wash out period during which all traces of the tested product are allowed to disperse from the test site.

Because of its controversial nature, the use of the Draize test in the U.S. and Europe has declined in recent years and is sometimes modified so that anaesthetics are administered and lower doses of the test substances used. Chemicals already shown to have adverse effects *in vitro* are not currently used in the Draize test, thereby reducing the number and severity of tests carried out. 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 at the grading intervals of 1, 24, 48 and 72 h. The reactions, defined as erythema and edema, were evaluated according to the scoring system for skin reactions as eye visible (**Table 20**). The Mexameter® MX 18 is a supplement device used for the confirmation of the erythema index (redness) in rabbit skin.

The Score of Primary Irritation was calculated for each rabbit. Scores for erythema and edema were summed and divided by the number of the observations for the treated sites. The SPI for the control sites were calculated in the same fashion (Spira et al., 1974). The differences between the summary scores from the treated sites and the control sites were calculated as the following formula including PII = [(∑ *erythema grade at 24/48/72 h* + ∑ *edema grade at 24/48/72 h)/ 3* × *number of*

animals]. The Primary Irritation Index (PII) was calculated as the arithmetical mean of the SPI values of the six animals. The irritation degree was categorized as negligible, or slight, moderate or severe irritation based on the PII (**Table 21**).

Table 20 Classification system for skin reactions (Spira et al., 1974)

Reaction	Score
Erythema	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to eschar formation	4
Edema	
No edema	0
Very slight edema (barely perceptible)	1
Well-defined edema (edges of the area well defined by definite raising)	2
Moderate edema (raising approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond the area of exposure)	4

Table 21 Response categories of irritation in rabbit (Spira et al., 1974)

Category	Primary Irritation Index (PII)
Negligible	0 – 0.4
Slight irritation	0.5 – 1.9
Moderate irritation	2 – 4.9
Severe irritation	5 – 8

1.4.8.2 Performance test in human volunteers

A. Skin elasticity measurement

The age-related decrease of skin elasticity results in larger fatigue of adult skin than young skin after applying multiple stresses at one and

the same anatomic region (Dobrev, 2005). The measuring principle is based on the suction method. Negative pressure is created in the device and the skin is drawn into the aperture of the probe, Cutometer (MPA 580, Courage and Khazaka, Koln, Germany). 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 transmitter to receptor. The light intensity varies due to the penetration depth of the skin. The resistance of the skin to be sucked up by the negative pressure (firmness) and its ability to return into its original position (elasticity) are displayed as curves at the end of each measurement.

The mechanical properties of the epidermis were determined equipped with 2 mm measuring probe. The time/strain mode was used with a 5-s application of a constant negative pressure of 450 mbar, followed by a 5-s relaxation period. A typical skin deformation curve is illustrated in (**Figure 33**). The curves of the obtained skin deformation values were analyzed using the software of Cutometer MPA 580 with R parameters as listed in **Table 22**.

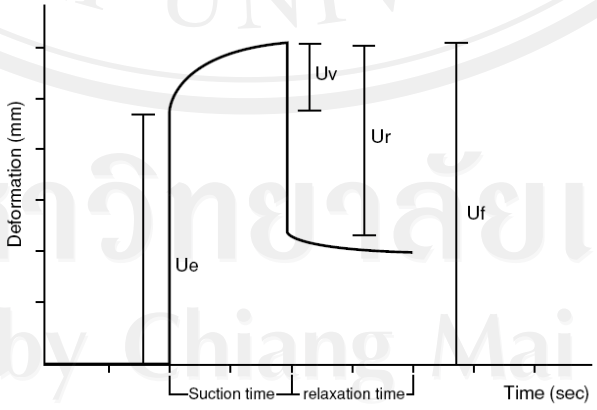


Figure 33 Skin deformation curve obtained with Cutometer. Cutometer parameters: Ue is the elastic deformation of the skin due to the application of stress (vacuum or

torque) by the instrument; Uv is the visco-elastic creep occurring after the elastic deformation; Uf is the total extensibility of the skin; Ur is the elastic deformation recovery due to stress removal; Ua is the total deformation recovery at the end of the stress-off period; R is the amount of deformation not recovered by the end of the stress-off period (Wickett, 2001)

Table 22 Definitions of skin elasticity indices by Cutometer (Dobrev, 2005)

Index	Expression	Point of measurement
R0	Uf	Length of the skin when elongated
R1	Uf-Ua	Length of the skin after elongation and constriction
R2	Ua/Uf	Recovery ratio of the skin length
R3	Uf	Length of the skin when elongated at second time
R4	Uf-Ua	Length of the skin after elongation and constriction at second time
R5	Ur/Ue	Ratio of elasticity part when elongated and during constriction
R6	Uv/Ue	Ratio of viscosity and elasticity part when elongated
R7	Ur/Uf	Ratio of elasticity part during constriction
R8	Ua	Length of skin constriction after first elongation
R9	Uf-Uf	Difference of skin length between first and second elongation

B. Skin roughness measurement

Estimation of skin smoothness and wrinkles are 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. Visiometer, a high-resolution UVA camera, is a tool for skin roughness evaluation which is a standardized non-contact method (Visioscan VC98, Courage and Khazaka Electronic GmbH, Cologne, Germany). It has been used for the evaluation of the skin surface parameters in cosmetic treatments. Digital analysis of

the gray level distribution of the skin's micro-relief was used to determine the roughness parameter. The images show the structure of the skin and the level of dryness. The camera features a high resolution black/white video sensor and a ring shaped UV-A light source (proven to present no hazard to normal human skin) for uniform illumination of the skin.

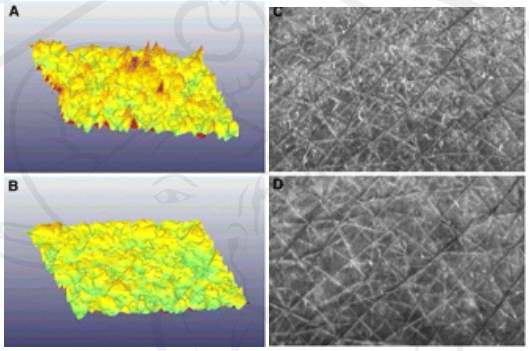


Figure 34 Examples of skin surface profiles of women evaluated with the surface evaluation of living skin method at week 0 (A) and after 12 weeks of consuming high flavanol cocoa beverages (B). The corresponding top view of the skin is shown in the lower photographs (Heinrich et al., 2011).

C. Skin hydration measurement

Environmental, physical, and nutritional alterations can modify epidermal structure and function. Techniques to characterize the skin's barrier function include a number of noninvasive methods to measure moisture content and loss through the skin surface (Barel et al., 1995). A method that has received some attention is assessment of the integrity of the skin barrier to insensible water loss. This technique is performed by measuring transepidermal water loss (TEWL) through the epidermal surface. The TEWL value is a measure of the rate of water lost through

the skin (in g/h·m2) and is an estimate of the skin's ability to retain moisture. Another technique is corneometry which determines the capacitance of the skin due to its behavior as a dielectric medium and assesses a 10–20 μm thickness of the stratum corneum. Although it is a measure of the water content of the skin, it is only an indirect measure of barrier function. Nonetheless, it can be related to the extent of hydration under various physiologic conditions in response to injury, metabolic phenomena, or topical therapies. Values reported for human skin evaluations differ depending on the body site that is studied (Blichmann et al., 1988; Pinnagoda et al., 1995; Watson et al., 2002).

Table 23 Parameters used in the assessment of skin visioscan® VC98

Parameter of skin roughness	Description
R1: skin roughness	The difference between the highest and the lowest gray values which the program cut the line into five equal parts
R2: average roughness	The average of the five maximum distance of R1
R3: maximum roughness	The biggest value of those five parts
R4: smoothness depth	The difference between average and base line
R5: arithmetic average roughness	The mean difference between baseline and profile

* The units of skin roughness parameter are micrometer (CK electronic GmbH, 2005).

D. Skin erythema and pigmentation measurements

The erythema (redness) is a signal of the sunburned skin and also indicated to immune system and the healing process is active. Melanogenesis (tanning) is the second pigmentary response, which is the development of darker skin on exposed regions of the UVR exposures. 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 reflection at three specific wavelengths which are 560, 650 and 710, corresponding to the output of the light-emitting diodes used for illumination. When the quantity of emitted light is defined, the quantity of light absorbed by the skin can be calculated (Dolotov et al., 2004).

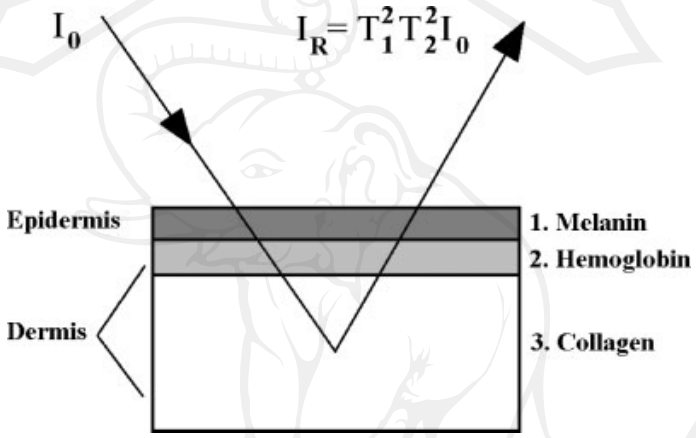


Figure 35 The reflectance of the simplified three-layer skin model. In the epidermis (layer 1), light is strongly absorbed by melanin and the non-absorbed part reaches the hemoglobin-rich papillary dermis (layer 2). The remaining part of the light is then diffusely reflected by dermal collagen (layer 3) (Dolotov et al., 2004)