CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problems

Thailand is a country with plentiful herbs and plants existing throughout the nation. As the leading of natural resource growers, Thailand gains knowledge over years, and unrivalled expert for the natural ingredient knowledge. Thailand offers wide range of herbal plant biodiversity along with the ancient knowledge of Thai traditional herbal medicines. Inspired by natural environments, implementation of natural herbs used as cosmetic ingredients is increased dramatically. However, many people still give trust and faith to many entrepreneurs, which specialize in creating active ingredients from herbs and natural source raw materials. Many business units, therefore, continue to provide natural source innovative products, formulation know-how and concepts for both domestic and international markets. At present, many Thai people consume more natural cosmetic products than synthetic cosmetic products. Some products are imported from abroad. Due to the fact that plants exist throughout Thailand, this causes natural cosmetic products to be more affordable and preferable than the synthetic cosmetics (Arad and Yaron,1992). Consequently, the export figure of synthetic cosmetic ingredients and products decreases dramatically. Many entrepreneurs work with customers more closely on exclusive concepts, actives, extracts and plant complexes. To be more competitive, they have increased their specialization in research and development of natural plant actives, extracts and essential oil. Since people pay more attention on boosting appearance and health of

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body and skin, the superb commercial opportunities in this fast growing natural cosmetic segment will be very interesting.

Certainly, this will truly enforce entrepreneurs to focus on the business potential of the fast-growing natural cosmetic market as well as natural cosmetic formulation and innovation. Thailand uses various herbs to prepare extract and essential oil, but only a few from flowers. The flowers used to prepare the extract should be unique and can be developed for commercial plantation.

In Thailand, one factory which has manufactured essential oils and natural extracts is the Thai China Flavours and Fragrances Industry Co., Ltd (TCFF) at Amphur Lad Bualuang in Phra Nakhon Si Ayutthaya. The essential oils and extracts that the company has produced are from *Zingiber Cassumunar* Roxb. (Phlai), *Cymbopogon citratus* Stapf (Lemon Grass), *Ocimum basilicum* L. (Sweet basil), *Ocimum sanctum* L. (Holy basil), *Citrus aurantifolia* (Christm.) Swingle (Common lime), *Piper betle* L. (Betel leaf Vine), *Curcuma longa* L. (Turmeric), *Zingiber officinale* Roscoe (Ginger) , *Boesenbergia rotunda* (L.), Mansf. (Finger root). The interested flowers of TCFF to produce essential oil and extracts to be used in cosmetics are *Millingtonia hortensis* (Cork Tree), *Gardenia jasminoides* J.Ellis (Cape Gardinia) and *Mimusops elengi* (Bullet Wood).

For applications of essential oil and extracts in cosmetic formulations, besides adding antioxidants to protect the constituents in essential oils and extracts from deterioration, there are several approaches to solve this problem including the use of nanotechnology. Nanotechnology for cosmetics includes the application of bilayer vesicles, such as liposomes and niosomes. Niosomes are a novel delivery system for drugs and cosmetic substances, in which the substance is encapsulated in the vesicles.

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These vesicles are bilayer vesicles composed of non-ionic surface active agents. Niosomes are very small, and microscopic in size. Their sizes are usually in the nanometric scale. Although structurally similar to liposomes, they offer several advantages over them (Uchegbu and Florence,1995) for example, better stability and smaller particle size than liposomes (Manosroi et al.,2010). Niosomes have recently been shown to greatly increase transdermal drug delivery and also can be used as targeted drug delivery systems (Manosroi A. and Manosroi J., 2007). Niosomes are also advantageous for cosmetic delivery. Niosomes can protect the entrapped substances, such as natural extracts or oil not to be destroyed by environmental factors including UV and temperatures.

In this study, the three flowers including *Gardenia jasminoides* (Family : RUBIACEAE), *Millingtonia hortensis* (Family : BIGNONIACEAE) and *Mimusops elengi* (Family : SAPOTACEAE). Which have never been studied for biological activities especially the MMP-2 inhibition activity, were extracted and entrapped in niosomes. The two non-heated methods including hexane maceration and supercritical carbon dioxide fluid extraction were be used. The extracts selected from their *in vitro* cosmetic activities (including antioxidative activities and tyrosinase inhibition activity) and the extraction yields were entrapped in the developed niosomes and the physical and chemical stabilities and other characteristics of the entrapped niosomes were investigated.

1.2 Objective

The purpose of this study was to entrap the Thai flower extracts in niosomes for cosmetic uses.

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1.3 Scope of the study

1.3.1 Collection of raw materials : Collection of 3 Thai flowers including *M.hortensis*, *G. jasminoides* and *M. elengi.*

1.3.2 Preparation of the extracts: Extraction of the flowers using the two non-heated methods which were the hexane maceration and the supercritical carbon dioxide fluid at various conditions using ethanol as a co-solvent.

1.3.3 Determination of physico-chemical properties and phytochemicals of the extracts: The physicochemical properties including solubility, color, pH, refractive index and stabilily in acid, base, oxidizers (e.g.H_2O_2) and reducers (e.g.$FeCl_3$). The phytochemical test to determine the presence of phenol, alkaloid, tannin, etc. was investigated by the standard methods.

1.3.4 Biological activity determination : The biological activities for cosmetic applications of the extracts such as DPPH scavenging and human fibroblast stimulation activities using Sulforhodamine B (SRB) assay were performed in order to evaluate their benefits for cosmetic applications.

1.3.5 Skin irritation study : Skin irritation test of the extracts on the rabbit's skin by the closed patch test was performed, and the erythema or irritation on the skin was optically observed or by using a Mexameter.

1.3.6 Selection of the extracts : At least one extract of each flower (total 3 samples) was selected by the evaluation from the yields, physico-chemical properties, phytochemicals, biological activities and rabbit skin irritation test.

1.3.7 Chemical Identification: Identification of the three selected extracts was performed by GC-MS and the constituents in each extract were investigated.

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1.3.8 The development of the 3 selected flower extracts loaded in the proper niosomal formulations.

1.3.9 Cost estimation of the selected extracts and the selected loaded niosomal formulation was calculated.

1.4 Literature reviews

1.4.1 Thai Flowers

1.4.1.1 Introduction

In Thailand, essential oils and extracts are mostly from leaves, rhizomes, seeds and fruits, but seldom from flowers. Thailand is in the tropical region of the world which has the advantages of biodiversity. Infact, Thailand has many famous flowers with good identity and characteristic scents that can be used as raw materials for essential oil and extract for cosmetic uses. At present, there are many leading cosmetic companies that are dedicated to search for new flower extracts, which are the nature's resources in order to enhance the beauty of individual. The example flowers that are in the great need for cosmetic ingredients are *Gardenia jasminoides* (Family : RUBIACEAE), *Millingtonia hortensis* (Family : BIGNONIACEAE) and *Mimusops elengi* (Family : SAPOTACEAE). The biological activities of the extracts have not been extensively performed. Infact, three flowers are valuable to be investigated for their cosmetic applications because of their identities and medicinal use in many folklore medicines. The general information and their medicinal properties as well as various researches on these three flowers are the followings :

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1.4.1.2 *Gardenia jasminoides* (Figure 1)

Figure 1 The flowers of *G. jasminoides* (http://en.wikipedia.org/wiki/gardenia)

G. jasminoides (also known as *G. augusta*) is a shrub with greyish bark and dark green shiny leaves with prominent veins. The white flowers bloom in spring and summer and are highly fragrant.They are followed by small oval fruit (Gilman, Edward F.,1999). It originated in Asia and is most commonly found growing in Vietnam, Southern China, Taiwan, Japan and India. *G. jasminoides* fructus (fruit) is used in Traditional Chinese Medicine to "drain fire" and to treat certain febrile conditions. It significantly lowered serum interleukin-1 β(IL-1β) and tumor necrosis factor α (TNF-α) levels in rheumatoid arthritis rats. Its effect had a close relation with inhibitory development of rheumatoid arthritis in rats.

Gardenia herb has been used as an alternative drug for thousand years. *G. jasmineoides* is a folk medicine with pharmacological activities in China.(Tseng et al.,1995). Three major crocins were isolated from *G. jasminoides*, the crocins appeared to possess antioxidant activity when tested by *in vitro* antioxidant models,

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including anti-hemolysis, DPPH radical-scavenging and lipid peroxidation assays. The gardenia resin fraction (GRF) exhibited significantly stronger antioxidant activity than crocins and no correlation between the total crocin contents and the antioxidative function was revealed, which implied that ingredients other than crocins in Gardenia gave markedly strong antioxidant activity (Chen, et al.,2008). Nine compounds from Gardenia fruit extracts (GFE) were identified (3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, quercetin-3-rutinoside, crocetin di(b-gentiobiosyl) ester, and geniposide). Caffeoylquinic acids, dicaffeoylquinic acids, and 4-sinapoyl-5-caffeoylquinic acid were the dominant free radical scavengers in GFE, and their Trolox equivalent antioxidant capacities (TEAC) were determined. 3, 5-Dicaffeoylquinic acid was the most potent antioxidant with the TEAC value of 1.1 mM. In contrast, crocetin di(b-gentiobiosyl) ester and geniposide showed little ABTS scavenging activity (He,et.al.2010). However, a filamentous fungi strain, *Penicillium nigricans*, producing glucosidase was screened to transform geniposide in Chinese traditional medicine, *G. jasminoides* into genipin, a highly efficient anti-inflammatory and anti-angiogenesis compound used in the treatment of liver fibrosis (Xua,et.al.,2008). The concentration of geniposide in the fruit of *G. jasminoides* Ellis var.*grandiflora* Nakai is higher than that in *G. jasminoides* Ellis (Tsaia et al., 2002).

High-speed counter-current chromatography (HSCCC) was applied to the isolation and purification of geniposide from *G.jasminoides* (Zhou et al., 2005). Geniposide was successfully isolated from *G. jasminoides*. Geniposide directly bound Lipopolysaccharide (LPS) and neutralized LPS *in vitro*, and significantly protected the sepsis model mice. Therefore, geniposide could be a useful lead compound for anti-

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sepsis drug development (Zheng et al., 2010). Gardenia fruit (*G. jasminoides* is widely used as a natural food colorant and a traditional Chinese medicine for the treatment of hepatic and inflammatory diseases. ''Gardenia yellow'' is a natural food colorant which is extracted by ethanol from gardenia fruit. Gardenia yellow and its components were found not to be mutagenic in the Salmonella reverse mutation assay (Ozakia et al., 2002). **Table 1** shows the chemical composition found in the extract of *G.jasminoides* flowers (Hattori, et al., 1978; Wang, et al., 1979; Guo, et al 1991).

1.4.1.3 *Millingtonia hortensis* (Figure 2)

Figure 2 The flowers of *M. hortensis*

From the dried flowers of *M. hortensis,* nine cyclohexylethanoids including four glucosides were isolated along with 12 related known compounds (Hase et al., 1995). From the flower buds of *M. hortensis, an* unusual glucosidal alkaloid was isolated in the diastereomeric form. Its structure has been established by the chemical and spectroscopic methods (Hase et al., 2006). **Table 2** shows The chemical compositions found in *M. hortensis* flowers (Nair et al, 1992 ; Chulasiri et al. 1992).

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers

CHEMICAL NAME	CHEMICAL GROUP
ACETIC ACID	ALKANE TO C4
AMYL BENZOATE	BENZENOID
ANTHRANILATE,METHYL	ALKALOID-MISC
BENZALDEHYDE	BENZENOID
BENZOATE,ETHYL	BENZENOID
BENZOIC ACID	BENZENOID
BENZOIC ACID METHYL ESTER	BENZENOID
BENZYL ACETATE	BENZENOID
BENZYL ALCOHOL	BENZENOID
BENZYL BENZOATE	BENZENOID
BENZYL BUTYRATE	BENZENOID
BENZYL CAPROATE	BENZENOID
BENZYL CROTONATE	BENZENOID
BENZYL TIGLATE	BENZENOID
BENZYL-2-METHYL-BUTYRATE	BENZENOID
BORNEOL-6-O-BETA-D-XYLOPYRANOSYL-BETA-D-GLUCOPYRANOSIDE	MONOTERPENE
BULNESOL	SESQUITERPENE
BUTAN-1-OL,2-METHYL	ALKANOL TO C4
BUTRIC ACID,2-METHYL	ALKANE TO C4

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
BUTYL,2-METHYL: 2-METHYL-BUTYRATE	ALKANE TO C4
CADINENE,ALPHA	SESQUITERPENE
CAPROATE,METHYL	LIPID
CAPROIC ACID	LIPID
CAPRYLIC ACID	LIPID
CARVEOL	MONOTERPENE
CINNAMYL ALCOHOL	PHENYLPROPANOID
CINNAMYL TIGLATE	PHENYLPROPANOID
CINNAMYL-2-METHYL-BUTYRATE	PHENYLPROPANOID
CITRONELLAL,HYDROXY	MONOTERPENE
CITRONELLOL	MONOTERPENE
CRESOL,PARA	BENZENOID
DECALACTONE,GAMMA	MISC LACTONE
DEC-CIS-7-ENOATE,5-HYDROXY ETHYL	LIPID
DODECALACTONE,GAMMA	MISC LACTONE
ELEMENE	SESQUITERPENE
ELEMOL	SESQUITERPENE
ETHANOL,2-PHENYL	BENZENOID
ETHYL CAPROATE	LIPID
ETHYL-2-METHYL-BUTYRATE	ALKANOL TO C4

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
EUGENOL,ISO	PHENYLPROPANOID
FARNESENE,ALPHA	SESQUITERPENE
FARNESOL	SESQUITERPENE
GARDENIC ACID	TRITERPENE
GARDENOLIC ACID B	TRITERPENE
GERANIC ACID,CIS-TRANS	MONOTERPENE
GERANIOL	MONOTERPENE
GERANYL BENZOATE	MONOTERPENE
GERANYL-LINALOOL	DITERPENE
GUAIACOL	BENZENOID
GUAIACOL,4-METHYL	BENZENOID
GUAIOL	SESQUITERPENE
HEPT-2-EN-6-ONE, 2-METHYL	MONOTERPENE
HEPTALACTONE, GAMMA	MISC LACTONE
HEPTAN-1-OL	ALKANE
HEPTYL BENZOATE	BENZENOID
HEXAN-1-AL	ALKANE
HEXAN-1-OL	ALKANE
HEX-CIS-3-EN-1-OL	ALKENE
HEX-CIS-3-EN-1-OL BENZOATE	BENZENOID
HEX-CIS-3-EN-1-OL BUTYRATE	ALKENE

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
HEX-CIS-3-EN-1-OL FORMATE	ALKENE
HEX-CIS-3-EN-1-OL ISO-BUTYRATE	ALKENE
HEX-CIS-3-EN-1-OL PROPIONATE	ALKENE
HEX-CIS-3-EN-1-OL TIGLATE	ALKENE
HEX-CIS-3-EN-1-OL-2-METHYL-BUTYRATE	ALKENE
HEX-CIS-3-EN-1-OL-HEX-CIS-3-ENOATE	LIPID
HEX-CIS-3-ENOATE,ETHYL	LIPID
HEX-CIS-3-ENOATE,METHYL	LIPID
HEX-CIS-3-ENOIC ACID	LIPID
HEX-CIS-3-ENOL ACETATE	ALKENE
HEX-TRANS-2-EN-1-OL	ALKENE
HEXYL ACETATE	ALKANE
HEXYL BENZOATE	BENZENOID
HEXYL TIGLATE	LIPID
HEXYL-2-METHYL-BUTYRATE	ALKANE
INDOLE	INDOLE ALKALOID
ISOAMYL ACETATE	ALKANOL TO C4
ISOAMYL ALCOHOL	ALKANOL TO C4
ISOAMYL BENZOATE	BENZENOID

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
JASMIN LACTONE	MONOTERPENE
LAURIC ACID	LIPID
LILAC ALCOHOL	MONOTERPENE
LILAC ALDEHYDE	MONOTERPENE
LINALOOL	MONOTERPENE
LINALOOL ACETATE	MONOTERPENE
LINALOOL OXIDE	MONOTERPENE
LINOLEIC ACID	LIPID
LINOLEIC ACID ETHYL ESTER	LIPID
LINOLEIC ACID METHYL ESTER	LIPID
LINOLENIC ACID	LIPID
LINOLENIC ACID ETHYL ESTER	LIPID
LINOLENIC ACID METHYL ESTER	LIPID
MYRISTIC ACID	LIPID
NEROL	MONOTERPENE
NEROLIDOL	SESQUITERPENE
NON-3-ENOIC ACID	LIPID
NONALACTONE, GAMMA	MISC LACTONE
OCIMENE, CIS:	MONOTERPENE

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
OCIMENE, TRANS:	MONOTERPENE
OCIMENOL	MONOTERPENE
OCT-1-EN-3-OL	ALKENE
OCTALACTONE,GAMMA	MISC LACTONE
OCTAN-1-OL	ALKANE
OCTYL BENZOATE	BENZENOID
OCTYL TIGLATE	ALKANE
OLEIC ACID	LIPID
OLEIC ACID ETHYL ESTER	LIPID
OLEIC ACID METHYL ESTER	LIPID
PALMITIC ACID	LIPID
PALMITIC ACID ETHYL ESTER	LIPID
PALMITIC ACID METHYL ESTER	LIPID
PALMITOLEIC ACID	LIPID
PENTADECANOIC ACID	LIPID
PHENOL, 2-ALLYL	BENZENOID
PHENOL, 4-ALLYL	BENZENOID
PHENYLACETALDEHYDE	BENZENOID
PHENYLACETATE, ETHYL	BENZENOID
PHENYLACETATE, METHYL	BENZENOID

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Table 1 The chemical compositions found in the extract of *G. jasminoides* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
PHENYLETHYL, 2: ACETATE	BENZENOID
PHENYLETHYL, 2: ALCOHOL	BENZENOID
PHENYLETHYL, 2: BENZOATE	BENZENOID
PHENYLETHYL, 2: TIGLATE	BENZENOID
PROPAN-1-OL, 3-PHENYL	PHENYLPROPANOID
ROSE OXIDE	OXYGEN HETEROCYCLE
SALICYLIC ACID METHYL ESTER	BENZENOID
SQUALENE	TRITERPENE
STEARIC ACID	LIPID
STEARIC ACID ETHYL ESTER	LIPID
STEARIC ACID METHYL ESTER	LIPID
STYROL ACETATE	PHENYLPROPANOID
TERPINEOL, ALPHA	MONOTERPENE
TIGLATE, ETHYL	ALKANE TO C4
TIGLATE, METHYL	ALKANE TO C4
TIGLIC ACID	MONOTERPENE
TIGLIC ACID METHYL ESTER	MONOTERPENE
UNDECALACTONE, GAMMA	MISC LACTONE
VANILLIN	BENZENOID

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Table 2 The chemical compositions found in the extract of *M hortensis* flowers

CHEMICAL NAME	CHEMICAL GROUP
APIGENIN-7-O-GLUCURONIDE	FLAVONOID
CIRSIMARITIN	FLAVONOID
CORNOSIDE	QUINOID
COUMAROYL, PARA: GLUCOSIDE	PHENYLPROPANOID
CYCLOHEXANE-1-4-DIOL,TRANS-1-(2'-HYDROXY-ETHYL)	ALICYCLIC
EUGENOL, ISO: BETA-D-GLUCOSIDE	PHENYLPROPANOID
HALLERIDONE	OXYGEN HETEROCYCLE
HISPIDULIN	FLAVONOID
HISPIDULIN-7-O-BETA-D-GLUCOSIDE	FLAVONOID
HISPIDULIN-7-O-GLUCURONIDE-METHYL ESTER	FLAVONOID
HISPULIDIN	FLAVONOID
HORTENSIN	FLAVONOID
PECTOLINARIGENIN	FLAVONOID
PECTOLINARIGENIN	FLAVONOID
PECTOLINARIGENIN	FLAVONOID
PHENYL)-ETHYL-GLUCOSIDE,2-(3-4-DIHYDROXY:	BENZENOID
SALIDROSIDE	BENZENOID
SCUTELLAREIN	FLAVONOID

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Table 2 The chemical compositions found in the extract of *M. hortensis* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUP
SCUTELLEREIN GLUCURONIDE	FLAVONOID
TRANS-1-(2-HYDROXYETHYL)CYCLOHEXANE-1,4-DIOL	FLAVONOID
VERBASCOSIDE	PHENYLPROPANOID

1.4.1.4 *Mimusops elengi* (Figure3)

Figure 3 The flowers of *M. elengi*

M. elengi is a medium-sized evergreen tree found in the tropical forests in South Asia, Southeast Asia, and Northern Australia. Its English common names include Spanish Cherr, Medlar, and Bullet wood. *M. elengi* with white flowers and the corolla preserves its fragrance even after drying. A comparative analysis of the fragrance obtained from *M. elengi* flowers by different methods including water soluble volatiles, hexane extract and liquid CO_2 extract were performed. The extracts were analyzed by GC and GC/MS. The chemical compositions of the extracts

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obtained were rich in bezenoids (61.7%), having phenyl ethyl alcohol (23.6–32.5%) as the major compound. The shade dried flowers were extracted by liquid CO_2 and the extract contained high percentage of the waxy materials (59.6%). The recovery of water soluble volatiles from the distilled water was carried out by partition with diethyl ether. The diethyl ether extract contained polar compounds (oxygenated terpenoids and benzenoids) and very few percentages of the waxy materials. It was observed that the liquidCO_2 extract of the fresh flowers, which was free from the solvent residue, was organoleptically superior in comparison to the extracts obtained by the conventional processes (Rout et al., 2010). Two medicinally important seed oils of *M. elengi* and *Parkinsonia aculeata* were analyzed for fatty acids distribution pattern in triacylglycerols using the pancreatic lipase hydrolysis method. The seed oils contain high percentage of unsaturated fatty acids (*M. elengi* 64.8% and *P. aculeata* 82.7%). The lipolytic data revealed that linoleic acid dominates at 2-position of triacylglycerols of all seed oils. *M. elengi* contains erucic acid in small amount (0.3%) (Sharma et al., 2009). **Table 3** shows the chemical compositions found in the extracts of *M. elengi* flowers (Wong, 1994 ;and Gupta , 1976).

The extraction and characteristics as well as the entrapment in niosomes of the extracts from the flowers of the three plants including *G. jasminoides, M. hortensis and M. elengi* have never been performed. Therefore, this research work has prepared the extracts from the flowers of these three plants by the proper methods. The characteristics and physico-chemical stability as well as their *in vitro* cosmetic activities of the extracts have been compared. The selected extracts were entrapped in a proper niosomal formulation and their potential for cosmetic applications was evaluated.

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Table 3 The chemical compositions found in the extract of *M. elengi* flowers

CHEMICAL NAME	CHEMICAL GROUPS
ANETHOLE,TRANS	PHENYLPROPANOID
ANISATE,METHYL	BENZENOID
ANISOLE,PARA-METHYL	BENZENOID
BENZALDEHYDE	BENZENOID
BENZOATE,BENZYL	BENZENOID
BENZOATE,ETHYL	BENZENOID
BENZOATE,METHYL	BENZENOID
BENZYL ACETATE	BENZENOID
BENZYL ALCOHOL	BENZENOID
BENZYL TIGLATE	BENZENOID
BUTAN-2-ONE,3-HYDROXY-4-PHENYL	BENZENOID
BUTANE-2-3-DIONE,1-PHENYL	BENZENOID
CINNAMALDEHYDE,TRANS	PHENYLPROPANOID
CINNAMIC ACID ETHYL ESTER	PHENYLPROPANOID
CINNAMIC ACID METHYL ESTER	PHENYLPROPANOID
CINNAMYL ACETATE,TRANS	PHENYLPROPANOID
CINNAMYL ALCOHOL,TRANS	PHENYLPROPANOID
CRESOL,PARA	BENZENOID
DAUCOSTEROL	STEROID
DECANOIC ACID ETHYL ESTER	LIPID
DODECANE	ALKANE

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Table 3 The chemical compositions found in the extract of *M. elengi* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUPS
DODECANOIC ACID ETHYL ESTER	LIPID
ESTRAGOLE	PHENYLPROPANOID
ETHANOL,2-4-METHOXY-PHENYL	BENZENOID
ETHYL BUTANOATE	ALKANOL TO C4
ETHYL HEXANOATE	ALKANOL TO C5
ETHYL PENTANOATE	ALKANOL TO C6
ETHYL-2-METHYLBUTANOATE	ALKANOL TO C7
EUGENOL	PHENYLPROPANOID
FARNESENE,ALPHA:TRANS-TRANS	SESQUITERPENE
GERANYLACETONE	SESQUITERPENE
GUAICOL,PARA-METHYL	BENZENOID
HEPT-4-ENOIC ACID ETHYL ESTER	ALKENE
HEPTAN-1-OL	ALKENE
HEX-3-CIS-ENOATE ETHYL ESTER	ALKENE
HEXADECANE	ALKENE
HEXAN-1-OL	ALKENE
HEXANAL	ALKENE
HEX-CIS-3-EN-1-OL ACETATE	ALKENE
HEX-TRANS-2-EN-1-AL	ALKENE
HEX-TRANS-2-EN-1-OL	ALKENE

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Table 3 The chemical compositions found in the extract of *M. elengi* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUPS
HEX-TRANS-2-ENYL ACETATE	ALKENE
HEXYL ACETATE	ALKENE
LAVANDULOL	MONOTERPENE
LINALOOL	MONOTERPENE
LINOLEIC ACID ETHYL ESTER	LIPID
MALONIC ACID DIETHYL ESTER	ALKANE
NEROLIDOL	SESQUITERPENE
NONAN-1-AL	ALKANE
OCIMENE,BETA: TRANS:	MONOTERPENE
OXALIC ACID DIETHYL ESTER	ALKANE
PALMITIC ACID ETHYL ESTER	LIPID
PENT-3-EN-2-OL	ALKANE
PENTADECANE	ALKANE
PHENOL,4-ALLYL-2-6-DIMETHOXY:	PHENYLPROPANOID
PHENYLETHANOL,2:	BENZENOID
PHENYLETHYL ACETATE,2:	BENZENOID
PHENYLETHYL,2: BENZOATE	BENZENOID
SALICYLATE,METHYL	BENZENOID
SITOSTEROL,BETA	STEROID
SUCCINIC ACID DIETHYL ESTER	ALKANE

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Table 3 The chemical compositions found in the extract of *M. elengi* flowers (continued)

CHEMICAL NAME	CHEMICAL GROUPS
TETRADECANE	ALKANE
TETRADECANOIC ACID ETHYL ESTER	LIPID
TRIDECANE	ALKANE
XYLENE,ORTHO:	BENZENOID

1.4.2. Extraction processes

1.4.2.1 Enfleurage

It is the classical extraction method of flowers and aromatic botanicals for perfumery and aromatherapy, as well as cosmetics using cold fats or lard as the extractant. This process is suitable for flowers which continue to emit fragrance even after plucking. The odorless fat is spread on glass plates which are placed in the closed system. The flowers are put in the system and replaced by the fresh one everyday until the fat is saturated with essential oil. The saturated fat is dissolved in alcohol at 30 to 40 °C and then cooled to 5 to 10 °C. The fat is precipitated out. The filtrate is concentrated under vacuum to eliminate alcohol and the liquid residue is called absolute. This process consumes extensive time, labor and other steps to eliminate alcohol and fat. However, the essential oil from this method is superior aroma since it is a non-thermo labile process (Giacomo A.D. and Giacomo G.D. 2002).

1.4.2.2 Solvent extraction

This process uses hydrocarbon solvents such as hexane, petroleum

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ether, benzene, toluene, ethanol, isopropanol, ethyl acetate and acetone, etc., to extract the plant materials. The extraction is a diffusion process which the extract transfers from the plant materials to the surrounding solvents, known as leaching. The operating temperature and time of the extraction are specific to the nature of the botanical substances and the extraction devices. The concentrated solution is done by vacuum distillation. The residue, dark colored and waxy substance, is called concrete. The concrete is dissolved in alcohol at 30 to 40°C and then cooled to 5 to 10°C which the wax will precipitate out. After filtration, the filtrate is concentrated under vacuum to eliminate alcohol. The liquid residue is called absolute.

As described above, there are many steps involved in recovering good quality and quantity of fragrance and extracts from botanical substances. The fragrance may lose the top notes, have the variety of volatile compounds, and contain some undesirable impurities, depending on the polarity of the solvents. The thermal degradation, hydrolysis and alcoholysis may happen and could affect the quality and stability of the oils and extracts. The important process of this method is the solvent elimination from the extracts, because of the harmfulness of the residue solvent.

1.4.2.3 Supercritical carbon dioxide fluid extraction ($ScCO_2$)

When a gas is compressed to a sufficiently high pressure, it becomes liquid. If the gas is heated to a specific temperature at the specific pressure, the hot gas will become supercritical fluid. This temperature is called the critical temperature and the corresponding vapor pressure is called the critical pressure. The values of the temperature and pressure are defined as critical point which is unique to a given substance. These states of the substances are called supercritical fluid when both the temperature and pressure exceed the critical point values. This fluid now takes on

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several of gas and liquid properties. Supercritical fluid is the region where the maximum solvent capacity and the largest variations in solvent properties can be achieved with small changes in temperature and pressure. It offers very attractive extraction characteristics owing to its favorable diffusivity, viscosity, surface tension and other physical properties. The diffusivity of the fluid state is one to two orders of magnitude higher than those of the liquid state. The diffuseness facilitates rapid mass transfer and faster completion of extraction than the conventional liquid solvents. The low viscosity and surface tension enable it to easily penetrate the botanical materials from which the active components are extracted. The gas-like characteristics of the supercritical fluid provide ideal conditions for the extraction of solutes giving a high degree of recovery in a short period of time. The most desirable supercritical fluid solvent for extraction of natural products is carbon dioxide (CO_2). It is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly, and generally regards as a safe solvent. In the supercritical fluid processing with CO_2, there is no solvent residue in the extract, because it becomes gas in the ambient condition. Its near-ambient critical temperature, 31.1 °C, makes it ideally suitable for thermolabile natural product extraction. Due to its low latent heat of vaporization, low energy input is required for the extraction separation system. The $ScCO_2$ produces the most natural smelling extracts, since the hydrolysis does not occur in the process. The advantages of the $ScCO_2$ technique are well known by now and it is often regard as an alternative to the classical methods. It has been established as an environmental friendly technique for separating essential oil from herbs or plants (Mukhopadhyay, 2000, Manosroi et. al, 2010a). Also, it has been widely applied for bilayer vesicle (liposomes/niosomes) preparation.

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1.4.3 Phytochemical determination

1.4.3.1 Alkaloids

Alkaloids are nitrogen-containing compounds widely distributed in different plants. Alkaloids are normally grouped on the basic of their ring system. Several common ring systems, including indolizidine- and quinolizidine-based systems and quinoline-, quinazoline-, and acridone-based systems were recently reviewed (Michael, 2004). Alkaloids in the form of plant extracts have been used for poisons, narcotics, stimulants and medicines for several thousand years. The common examples include caffeine, quinine, nicotine, cocaine, morphine and strychnine **(Figure 4)**. Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (potassiomercuric iodide solution) and gives cream colored precipitate while Dragendorff's reagent (solution of potassium bismuth iodide) gives an orange colored precipitate with alkaloids.

1.4.3.2 Flavonoids

Flavonoids have two benzene rings separated by a propane unit. They are generally water-soluble compounds The more conjugated compounds are often brightly colored. They are found in plants as their glycosides, which have complicated structure. The different classes within the group are distinguished by the additional oxygen-containing heterocyclic rings and hydroxyl groups. These include the chalcones, flavones, flavonols, flavanones, anthocyanins and isoflavones (**Figure 5**) (Williams and Grayer, 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).

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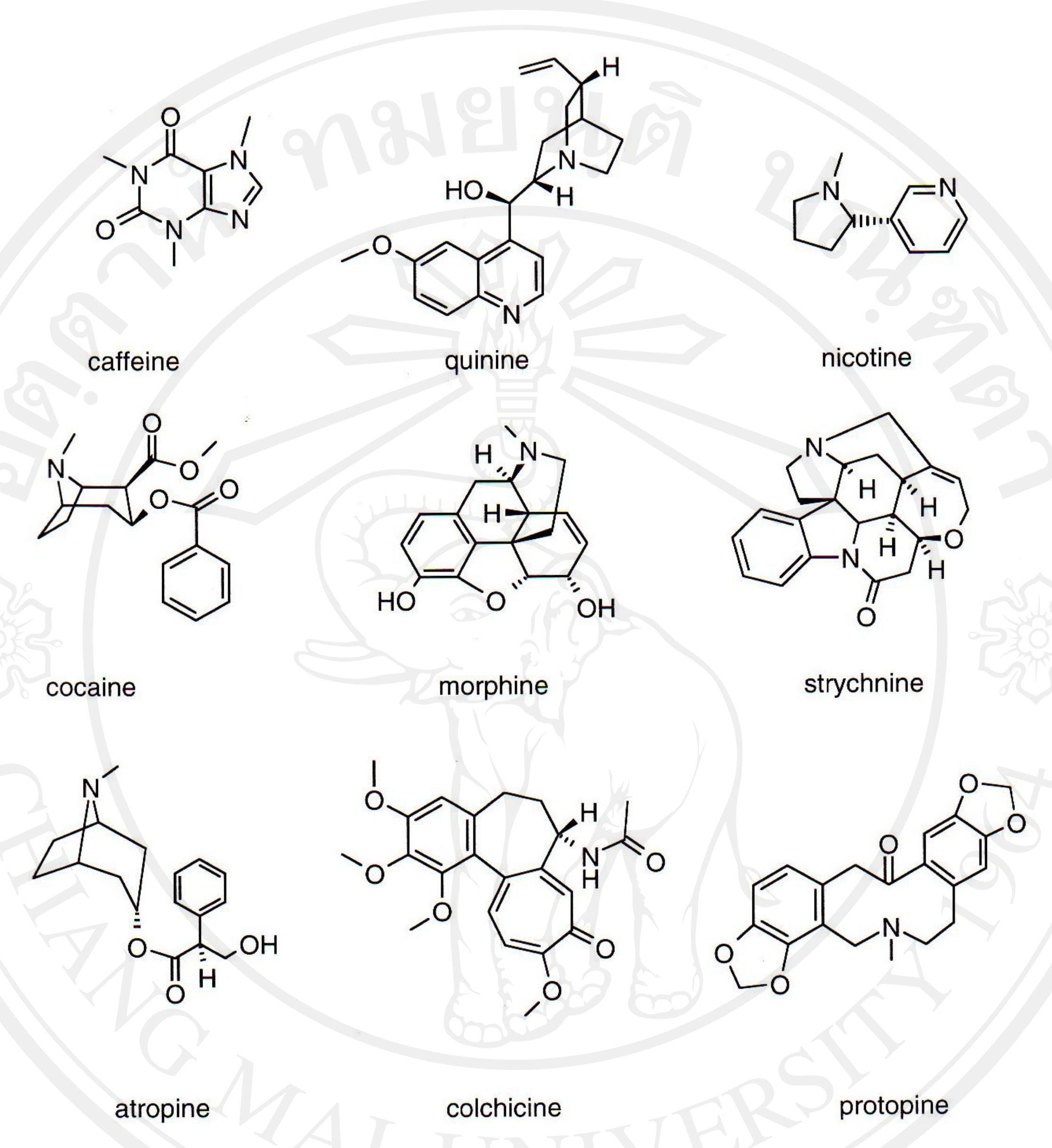

Figure 4 Chemical structures of some common alkaloids (Michael, 2004)

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1.4.3.3 Saponins

Saponins are high-molecular-weight triterpene glycosides, containing a sugar group attached to either a sterol or triterpene. Saponins are composed of two parts which are glycone (sugar) and aglycon or genin (triterpene). Typically, they have detergent properties, readily form foams in water, have a bitter

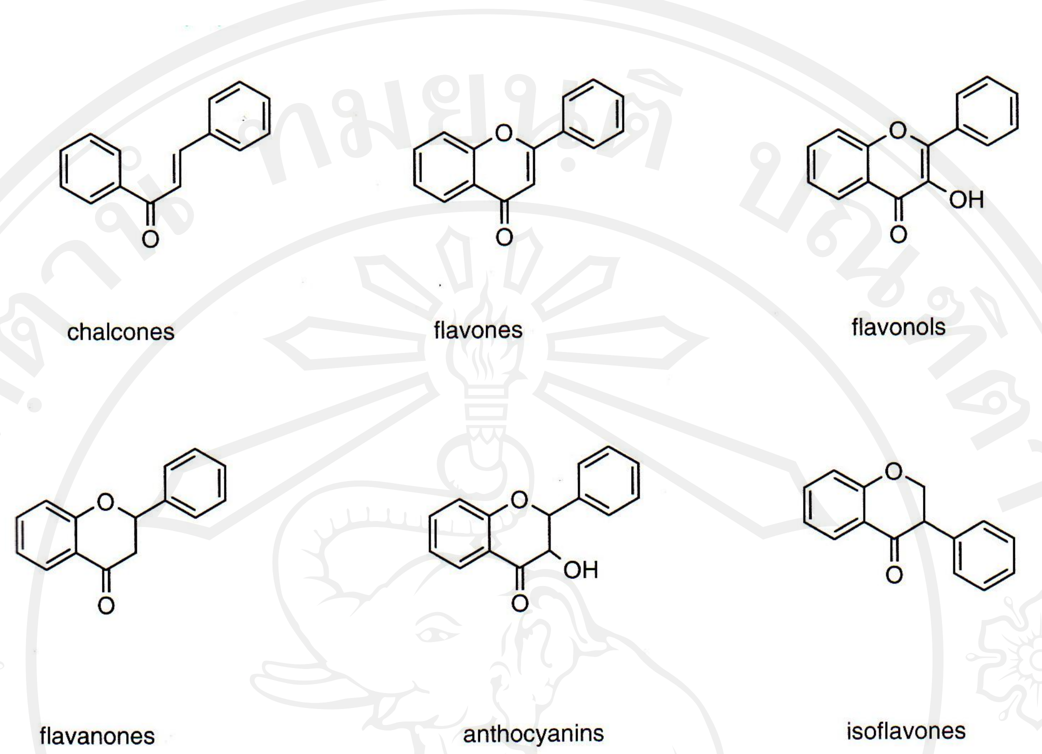

Figure 5 Chemical structures of flavoniod classes (Williams and Grayer, 2004)

taste, and toxic to fish. Many plants that contain saponins were historically used as soaps. These include Soaproot (*Chlorogalum pomeridianum*), Soapbark (*Quillaja saponaria*) and Soapnut (*Sapindus mukurossi*) (Hostettman & Marston, 1995). The aglycons may be triterpene, steroid or steroid alkaloid class. Representative saponins were presented in **Figure 6**.

1.4.3.4 Tannins

Tannins are water-soluble oligomers, rich in phenolic groups, capable of binding or precipitating water-soluble protein. The tannins, common to vascular plants, exist primarily within woody tissues, but can also be found in leaves, flowers or seeds. Plant tissues that are high in tannin contents have a highly bitter taste. Tannin may be divided into two groups which are either condensed tannins or

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Figure 6 Chemical structures of saponins (Hostettman & Marston, 1995)

sugar with one or more trihydroxy benzene carboxylic acids (gallic acid). These tannins give insoluble precipitates with albumin, starch or gelatin. This reaction with proteins is used industrially to convert animal skins into leather. Tannins produce different colors with ferric chloride (either blue, blue black, or green to greenish black) according to the type of tannin (Hagerman & Butler, 1989).

1.4.3.5 Reducing sugars

A reducing sugar is any sugar that, in a solution, has an aldehyde or a ketone group (**Figure 8**). This allows the sugar to act as a reducing agent, for example in the Maillard reaction and Benedict's reaction. A sugar is only a reducing sugar if it has an open chain with an aldehyde or a ketone group. Many sugars occur in a chain as well as in a ring structure. In solution, it is possible to have equilibrium between

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these two forms. The aldehyde can be oxidized via a redox reaction. The that reduces certain chemicals. Even though a chemical that causes this oxidation

proanthocyanidin from ***Sorghum***

proanthocyanidin from ***Eucalyptus***

corilagin

geraniin

Figure 7 Chemical structures of condensed (proanthocyanins) and hydrolysable (corilagin and geraniin) tannins (Hagerman & Butler, 1989)

becomes reduced. Thus, a reducing sugar is one ketone cannot be oxidized directly, a keto sugar may be converted to an aldehyde via a series of tautomeric shifts to migrate the carbonyl to the end of thechain. Reducing monosaccharides include glucose, fructose and galactose. Benedict's reagent and Fehling's solution are used to test for the presence of a reducing sugar. The reducing sugar reduces copper (II) ions in these test solutions to copper (I), which then forms a brick red copper (I) oxide precipitate.

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3, 5-Dinitrosalicylic acid is another test reagent that allows quantitative spectrophotometric measurement of the amount of the reducing sugar. Sugars having acetal or ketal linkages are not reducing sugars, as they do not have free aldehyde chains. Therefore, they do not react with any of the reducing-sugar test solutions. However, a non-reducing sugar can be hydrolysed using dilute hydrochloric acid. After hydrolysis and neutralization of the acid, the product may be a reducing sugar that gives normal reactions with the test solutions.

1.4.3.6 High performance liquid chromatography (HPLC)

Difficulties in quality control of phytochemical substances are due to the complexity of their structure, the small quantities of the compounds and the time-consuming of the extraction procedures. However, HPLC can provide both qualitative and quantitative information of the substances, which is usually difficult to obtain by other techniques.

High-pressure liquid chromatography, sometimes called high-performance liquid chromatography (HPLC), is a separation technique based on a solid stationary phase and a liquid mobile phase. Separations are achieved by partition, adsorption, ion-exchange process, depending upon the type of stationary phased used. HPLC has distinct advantages over gas chromatography for the analysis of organic compounds. Compounds to be analysed are dissolved in a suitable solvent and most separations take place at room temperature. Thus, most drugs being nonvolatile or thermally unstable compounds can be analysed without decomposition or the necessity of making volatile derivatives. Unique characteristics in HPLC separation are described as the followings :

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1 Separations are carried out on a given HPLC column using closed reusable columns.

2 Sample application can be precisely injected without difficulty either by using syringe injection or sample loop.

3 Solvent flow is achieved by using a high pressure pump, which gives the controlled and rapid flow of solvent resulting in reproducible operation with excellent accuracy.

4 Detection and quantitation are observed by continuous detector providing the continuous chromatograms without intervention by the operator.

Figure 8 Chemical structures of reducing forms of glucose (left) and fructose (right)

1.4.4 Nanovesicles

1.4.4.1 Classification of nanovesicles

Recently, numurous vesicular delivery systems have been exploited for enhanced transdermal uses. They are divided into 4 major groups including liposomes, niosomes, transfersomes and ethosomes (Lasic, 2001).

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A. Liposomes

Liposomes are microscopic vesicles composed of a membrane like- phospholipid bilayer surrounding aqueous compartments. It can entrap both hydrophilic and hydrophobic substances in their structure. The hydrophilic substances are embedded in aqueous part of the vesicles while hydrophobic or amphiphilic substances are solubilized and intercalated into the vesicular membrane. The occurrence of liposomes has been first described by Alec D. Bangham in 1965. They have demonstrated that phospholipids are able to aggragate spontaneously to spherical lipid vesicles in a water phase. However, phospholipids are not only form bilayer vesicles but other forms of self-aggregation such as spherical or hexagonal micelles, inverted micelles or planar bilayers can also occur. Israelachvili et al. (1985) have proposed the critical packing parameter (*CPP*) as a tool to predict the structure of lipids or amphiphilic compounds in aqueous solution. The critical packing parameter can be calculated from the following equation:

$$CPP = v / a_o l_c$$

Where, v is the molecular volume of the hydrophobic part, a_o is the optimum surface area of the head group and l_c is the critical length of alkyl chain.

Figure 9 shows the critical packing parameter of lipids and their structure formation.

As aforementioned, phospholipids are general constituents of liposomes. Crommelin and Schreier (1994) have distinguished phospholipids used for liposomal preparation into five groups as the followings:

(i) Phospholipids from natural sources: phosphatidyl choline (PC), phosphatidyl ethanolamine(PE), phosphatidylinositol (PI) and sphingomyelin (SPM)

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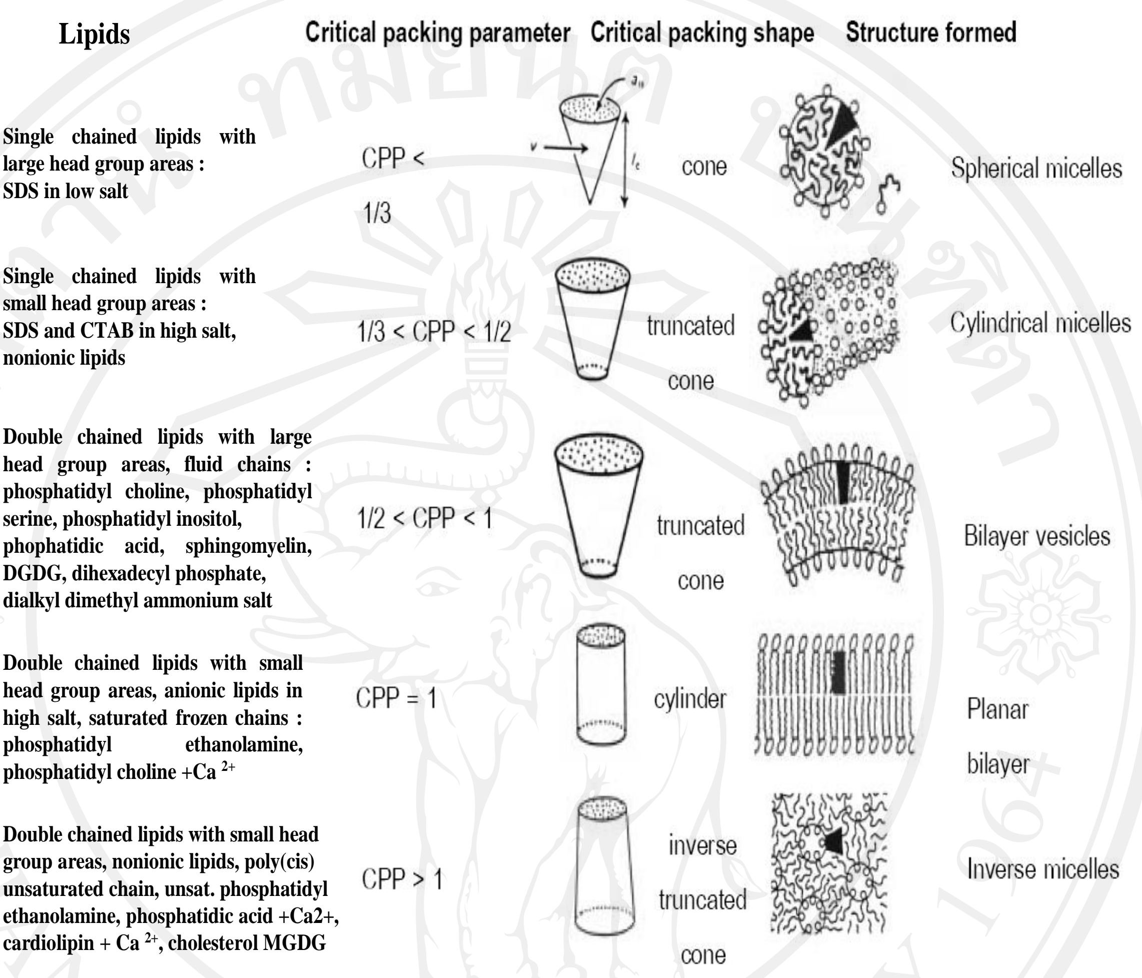

Figure 9 The critical packing parameter (*CPP*) of lipids and their structure form (DGDG: digalactosyl diglyceride and diglucosyl diglyceride; MGDG: monogalactosyl diglyceride and monoglucosyl diglyceride) (Israelachvili et al., 1983, 1985)

(ii) Modified natural phospholipids: partially or fully hydrogenated natural PCs, head group modification by phospholipase D for avoiding oxidative degradation

(iii) Semi-synthetic phospholipids: removal or replacement of the Original acyl chain of phospholipids for increasing stability

(iv) Fully synthetic phospholipids

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(v) Phospholipids with non-natural (head) groups: polyethylene glycol (PEG) linked to PE for the long circulating Liposomes, protein binding to phospholipid for immunoliposomes

Although liposomes nomenclature has not been standardized yet, liposomes have been classified and given specific acronyms by their structural properties or method of preparation (Crommelin and Schreier, 1994). The classifications of liposomes and the regularly used acronyms are shown in **Table 4.**

Up until now, liposomes have been studied in several applications including basic sciences, medicines, cosmetics, bioengineering and ago-food industries.

A.1 Liposomes in the basic sciences

The structure of liposomes or bilayer vesicles offers a model to understand the topology, shape fluctuations, phase behaviour, permeability, fission and fusion of biological membranes (Villalobo, 1991).

A.2 Liposomes in medicines (Lasic, 1995)

Liposomes have been used as drug delivery systems especially for parenteral, topical, and pulmonary route of administration. The advantages of liposomes as drug carriers are summarized as the followings:

(i) Liposomes can improve solubility of many lipophilic drugs such as porphyrins, amphotericin B, minoxidil, peptides or anthracyclines, and hydrophilic drugs such as doxorubicin or acyclovir.

(ii) Liposomes offer passive targeting to the cells of the immune system for several drugs such as antimonials, amphotericin B, porphyrins and vaccines, immunomodulators or (immuno) supressors.

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Table 4 Classifications of the liposome based on their structural properties and the preparation methods (Crommelin and Schreier, 1994)

Liposome types	Description
I. Based on structural properties	
MLV	Multilamellar large vesicles, size > 0.5 μm
OLV	Oligolamellar vesicles, size of 0.1-1 μm
UV	Unilamellar vesicles (all sizes)
SUV	Small unilamellar vesicles, size of 20-100 mm
MUV	Medium-sized unilamellar vesicles
LUV	Large unilamellar vesicles, size > 100 mm
GUV	Giant unilamellar vesicles, size > 1 μm
MVV	Multivesicular vesicles, size > 1 μm
II. Based on method of liposome preparation	
REV	Single or oligolamellar vesicles made by the reverse phase evaporation method
MLV-REV	Multilamellar vesicles made by reverse phase evaporation method
SPLV	Stable plurilamellar vesicles
FATMLV	Frozen and Thaw MLV
VET	Vesicles prepared by extrusion method
FPV	Vesicles prepared by French press
FUV	Vesicles prepared by fusion
DRV	Dehydration-rehydration vesicles

(iii) Liposomes can be sustained release system for many drugs for examples doxorubicin, cytosine arabinose, cortisones, biological proteins or peptides such as vasopressin.

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(iv) Liposomes have site-avoidance mechanism, for examples, the reduction of nephrotoxicity of amphotericin B and cardiotoxicity of doxorubicin liposomes.

(v) Liposomes can specify site targeting of many drugs by surface attached ligands (‘key and lock’ mechanism) such as anticancer, anti-infection and anti-inflammatory drugs.

(vi) Liposomes improve the transferation of hydrophilic, charged molecules such as chelators, antibiotics, plasmids, and genes into cells.

(vii) Liposomes improve the penetration of drug into tissues, especially dermal application, such as anaesthetics, corticosteroids and insulin.

A.3 Liposomes in cosmetics (Lasic, 1995)

Liposomes can be utilized in the delivery of ingredients in cosmetics. In addition, liposomes offer advantages because lipids are well hydrated and can reduce the dryness of the skin which is a primary cause of aging. Also, liposomes can supply lipid to the skin. Liposomes are also claimed to enhance permeability of cosmetic ingredients into the skin. Liposomal cosmetic products which are currently in the market are listed in **Table 5**.

A.4 Liposomes in bioengineering

Modern genetic engineering and gene recombinant technology are based on the delivery of genetic materials such as fragments of DNA, into various cells and microorganisms in order to alter their genetic code and force them to produce particular proteins or polypeptides. Nucleic acids used in gene transfer are large, with molecular weights of up to several million daltons, highly charged and hydrophilic and therefore are not easy to transfer across the cell membranes. Transfection has successfully performed using small unilamellar vesicles

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made from positively charged lipids such as dioleoyl-propyl-trimethylammonium (DOTMA) (Felgner et al., 1987). However, liposomes containing positively charged cholesterol give better transfection efficiencies at reduced toxicity (Gao and Huang, 1991). Thus, liposomes are expected to be useful tool in gene therapy.

Table 5 Liposomal cosmetic formulations currently in the market (Lasic, 1995)

Products	Manufacturers	Liposomes and key ingredients
Capture	Cristian Dior	Liposomes in gel with ingredients
Efect du Soleil	L'Or´eal	Tanning agents in liposomes
Aquasome LA	Nikko Chemical Co.	Liposomes with humectant
Symphatic 2000	Biopharm GmbH	Thymus extract, vitamin A palmitate
Natipide II	Nattermann PL	Liposomal gel for do-it-yourself cosmetics
Flawless finish	Elizabeth Arden	Liquid make-up
Formule Liposome Gel	Payot (Ferdinand Muehlens)	Thymoxin, hyaluronic acid
Nactosomes	Lancome (L'Or´eal)	Vitamins
Future Perfect Skin Gel	Estee Lauder	TMF, vitamins E, A palmitate, cerebroside ceramide, phospholipid
Inovita	Pharm/Apotheke	Thymus extract, hyaluronic acid, vitamin E
Eye Perfector	Avon	Soothing cream to reduce eye irritation

A.5 Liposomes in agro-food industries

Liposomes have become an attractive system in agro-food industries because they are composed of entirely acceptable food compounds. The sustained release system of liposomes can be used in various fermentation

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processes, for instances, the encapsulated enzymes in liposome in cheese making can shorten fermentation times and improve the quality of the product (Law and King, 1991). Besides fermentation improvement, liposomes are used in the preservation (Kirby, 1990). Moreover, biocides encapsulated in liposomes have shown superior action due to prolonged presence of the fungicides or herbicides.

B. Niosomes

Niosomes or non-ionic surfactants vesicles have been first reported as a carrier for cosmetics by Handjani-Vila and co-workers in 1979. Niosomes have been exploited as the alternatives to liposomes due to the lower cost, higher purity and more chemical stability of non-ionic surfactants than that of phospholipids. Several types of non-ionic surfactants such as polyglycerol alkyl ethers, glucosyl dialkyl ethers, crown ethers and polyoxyethylene alkyl ethers and esters have been used to prepare niosomal vesicles. The ability of surfactants to form a vesicles can be predicted by *CPP* the same as that of phospholipids in forming liposomes (Israelachvili et al. 1983). Uchegbu and Vyas (1998) have reported that the hydrophilic-lipophilic balance (HLB) of surfactants is a good indicator for the prediction of vesicle forming ability. For instances, sorbitan monostearate (Span) surfactants with HLB of 4 to 8 can form vesicle (Yoshioka et al., 1994), whereas Polysorbate 20 with HLB of 16.7 appears to be too hydrophilic to form a bilayer membrane (Santucci et al., 1996). However, nonionic surfactants with HLB > 6 can form bilayer vesicles by the addition of cholesterol (Lawrence, 1996). The vesicles formation ability, HLB values and chemical structure of some non-ionic surfactants are shown in **Table 6** (Manosroi et al., 2003). As aforementioned, niosomes have distinct advantages over conventional liposomes, and can serve as alternative drug

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Table 6 The vesicles formation ability, HLB values and chemical structure of some non-ionic surfactants (Manosroi et al., 2003)

Name	Chemical structure	HLB	Vesicle formation without cholesterol
Sorbitan monostearate (Span 60)		4.7	Yes
Polyoxyetheylene sorbitan monostearate (Tween 61)		9.6	No
Polyoxyethylene 2 stearyl ether (Brij 72)		4.9	Yes
Polyoxyethylene 4 lauryl ether (Brij 30)		9.7	No
Glyceryl monostearate (GMS)		3.8	Yes
Diglyceryl monolaurate (DGL)		6.7	No
Tetraglyceryl monolaurate (TGL)		10.0	No

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carriers. The potential of niosomes in drug delivery systems has been proven by several methods of administration (Blazek-Welsh and Rhodes, 2001) including intramuscular (Arunothayanun et al., 1999), intravenous (Pillai and Salim, 1999), oral (Rental et al., 1999), and transdermal route (Uchegbu et al., 1995). Niosomes have been shown to enhance absorption of some drugs across cell membranes (Lasic, 1993), to localize in targeted organs (Namdeo and Jain, 1999) and tissues (Baillie et al., 1986), and to elude the reticuloendothelial system (RES) (Gopinath et al., 2001). Moreover, the high encapsulation efficiencies of various drugs in niosomes have been reported including anti-cancer agents (Chandraprakash et al, 1993; Uchegbu et al., 1996; Paolino et al., 2008), anti-inflammatory agents (Reddy and Udupa, 1993), vaccine adjuvants (Brewer et al., 1996), diagnostic imaging agents (Erdogan, 1996) and protein or DNA (Ciotti and Weiner 2002).

C. Transfersomes

Transfersomes or flexible liposomes have first introduced in 1991 by Cevc as a special lipid vesicle which can penetrate efficiently through the pores that would be restriction for other particulates of comparable size. Transfersomes consist of phospholipids incorporated with edge activator (10-25%) and the small amount of ethanol (3-10%). Edge activator is often a single chain surfactant such as sodium cholate, Span 80, Tween 80 and dipotassium glycyrrhizinate. They destabilize lipid bilayers of the vesicles and increase deformability of the bilayer. The efficacy and safety of transfersomes in pharmaceutical applications have been investigated by several studies. Cevc and Blume (2001, 2003) have reported that transfersomes up to 500 nm can squeeze and pass through the polar channel of skin upon hydration gradient while conventional liposomes are too large to pass through

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the pores of less than 50 nm. They have also found that diclofenac in transfersomes give a longer anti-inflamatory effect and reach 10-times higher concentrations in the skin in comparison with the commercial hydrogel (Cevc and Blume, 2001). (Guo et al., 2000) have demonstrated that insulin incorported in flexible lecithin liposomes applied to mouse skin give hypoglycaemia effect, whereas conventional liposomes and insulin solution had no effect. El Maghraby et al. (2000a, 2000b) have demonstrated the superior skin permeation enhancement of estradiol by ultradeformable liposomal formulation (17-fold) in comparing to the conventional liposomes (9-fold). Rother et al. (2007) have reported that epicutaneous ketoprofen in transfersome is able to relieve pain associated with acute flare of knee osteoarthritis in comparing to oral celecoxib. Recently, Geusens et al. (2009) have presented that ultradeformable cationic liposomes can deliver siRNA into *in vitro* human primary melanocytes. Thus, transfersomes are expected to be one of the most effective non-invasive deliveries of many therapeutic agents across the skin.

D. Ethosomes

Ethosomes are lipid vesicles with high alcohol contents (up to 45%) capable of enhancing penetration to deep tissues and the systemic circulation (Touitou et al., 2000). Ethosomes have been reported to improve skin delivery of various drugs such as acyclovir, minoxidil, testosterone and ammonium glycyrrhizinate (Horwitz et al., 1999; Touitou et al., 2000; Ainbinde and Touitou, 2005; Paolino et al., 2005). Touitou et al. (2000) have proposed that ethanol may fluidizes the ethosomal lipids and stratum corneum bilayer lipids, thus allowing ethosomes to penetrate through the skin. Elsayed et al. (2006) have also studied the possible mechanisms of deformable liposomes and ethosomes for skin delivery of

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ketotifen inside and outside the vesicles under non-occlusive conditions. The results have indicated that deformable liposomes improve skin delivery of ketotifen by both the penetration enhancing effect and as intact vesicle permeation into the stratum corneum, while ethosomes are not able to improve skin delivery of the unentrapped ketotifen. However, the mechanisms of ethosomes on skin penetration enhancement have not been clearly understood.

1.4.4.2 Preparation of nanovesicles

The characteristics of nanovesicles including size, numbers of lamellae, charge, bilayer rigidity and behavior of the vesicles *in vitro* and *in vivo* can be varied by different preparation techniques and bilayer constituents. Thus, it is very important to clearly understand the principle of preparation techniques in order to get the proper vesicular formation.

A. **Ether injection method**This method based on the replacement of organic solvents by aqueous media. Briefly, the bilayer constituents are dissolved in organic solvent which is subsequently injected into aqueous phase. The organic solvent is then removed. During the removal of organic phase, vesicles are formed. The multilamellar vesicles (MLV), oligolamellar vesicles (OLV) and large unilamellar vesicles (LUV) will be obtained by this technique. Deamer and Bangham (1976) have prepared LUV liposomes by slow injection of ether or ether with methanol solutions of lipids into the water phase, then removal of organic solvents by evaporation either at elevated temperature or under reduced pressure. Ether injection has been used for the production of liposomes entrapped with plasmid DNA due to high aqueous volume of LUV and the absence of the sonication step thereby facilitating entrapment of macromolecules without loss of biological activity (Fraley et al., 1979). Pham et al.

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(2006) have reported the success of immuno-stimulating complexes (ISCOM) prepared by this method. Stable niosome entrapped with acyclovir by ether injection has been introduced by Rangasamy et al. (2008).

B. **Hand shaking method (thin film hydration technique)**

This method based on mechanical technique. It has been first described by Dingle et al. in 1978. Briefly, the thin films deposited from an organic solution on a glass wall is hydrated and shaked vigorously at temperature above the phase transition temperature (Tc) of bilayer constituents. Then, MLV will be obtained. Although hand shaking method are easy and quick technique for preparing vesicles, the entrapment volumn of vesicles is low. Hence, this method is not good for entrapping of water-soluble compounds (Perkins et al., 1988). Moreover, the broad size distribution of vesicles are usually obtained by this technique. Low pressure extrusion and ultrasonication are proposed to reduce and narrow down the size distribution of these vesicles (Jousma et al., 1987). Guo et al. (2000) have prepared conventional and flexible lecithin vesicles entrapped with lipohilic cyclosporine A by thin film hydration technique with sonication. Both vesicular formulations show small and narrow distribution in size. Koromila et al. (2006) have found that MLV type of liposomes prepared by thin film method give low entrapment efficiency for heparin- anticoagulating drug. Recently, Wang et al. (2009) have demonstrated the successful preparation of biodegradable polymer [Poly(3-caprolactone)-block-poly(ethyl ethylene phosphate)] vesicles by thin film hydration technique.

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C. Reverse phase evaporation method

This method based on the replacement of organic solvents by aqueous media. Briefly, the lipids are dissolved in water immiscible solvent (chloroform/ ether). Then, they are emulsified with water phase by sonication and controlled the removal of organic phase by evaporation. Reverse phase evaporation vesicles (REV) containing large unilamellar or oligolamellar vesicles will be formed by this technique (Szoka and Papahadjopoulos, 1978). Mertins et al. (2005) have prepared the nanovesicles containing soybean phosphatidylcholine (PC) - chitosan by reverse phase evaporation method. PC is dissolved in ethyl acetate, water or chitosan solutions in acetate buffer saline are then dropped into the solution to form a water in oil (w/o) emulsion, of which is sonicated yielding a homogeneous reverse micelles dispersion. The organic solvent is evaporated by a rotatory evaporator, giving a high viscous organogel. The organogel is reverted to nanovesicles after addition of water under shaking. The critical step in the REV preparation is the removal of organic solvent where w/o emulsion is formed. Vigorous shaking is needed to convert the emulsion to vesicular form (Cortesia et al., 2002).

D. Transmembrane pH gradient (inside acidic) drug uptake process (remote loading)

This method based on the pH adjustment. It has been first demonstrated by Nichols and Deamer in 1976. The drug should be able to change from uncharged species, which can diffuse across the membrane, to charged species that can not pass through membrane. The degree of ionization of drugs is dependent on their pKa and on the local pH (Clerc and Barenholz, 1998). Only amphipatic weak acids (for examples acetate) or bases (for examples ammonium) can be loaded into the

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vesicles by this method. Another requirement for remote loading is the driving force caused by the trans-membrane gradient, which will "pump" the drug from the external liposome medium into the liposome (Zucker et al., 2009). SUV and LUV will be obtained by this technique. To date, many drugs are remotely loaded successfully into liposomes using various gradients such as diclofenac, insulin (Hwang et al., 1999) bupivacaine (Grant et al., 2004), topotecan (Li et al., 2006), doxorubicin (Barenholz, 2007) and glucocorticosteroids, for examples beta methasone succinate, methyl prednisolone succinate (Metselaar et al., 2003; Avnir et al., 2008).

E. Formationof proliposomes and liposomes from proliposomes

The proliposomes - liposome method has been developed by Payne and co-workers (1986a). They deposited the lipids from an organic solvent on finely powdered sodium chloride or sorbitol. Upon hydration, liposomal dispersions are formed. Proliposomes are free-flowing particles composed of drug, phospholipids and a water-soluble porous powder that immediately form a liposomes upon hydration. MLV are usually formed by this technique. Moreover, the narrow size distribution of the reconstituted liposomes can be obtained by controlling of size of the porous powder in proliposomes (Payne et al, 1986b). Hwang et al. (1997) have indicated that transdermal delivery of nicotine is sustained by proliposomes. Ishikawa et al. (2004) have reported that high encapsulation efficiency (41.9%) of bovine serum albumin (BSA) is achieved using proliposomes with soy bean lecithin. Hiremath et al. (2009) have revealed that the proliposomes are successful in enhancing the permeation of exemestane in rat intestine, parallel artificial membrane permeability assay (PAMPA) and Caco-2 cell line model.

F. The bubble method

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This method based on the mechanical technique which be used for generating vesicles in one step without organic solvents or detergents and under low shear condition. Talsma et al. (1994) have produced liposomes at temperature above phase transition temperature (Tc) of lipids by hydration of phospholipids in a stream of bubbling inert gas for examples nitrogen, perfluoropropane through the aqueous phase. Liposomes are generated on the collapse of N_2 bubbles coated with phospholipid layers at water/ air interface. Due to the low shear force of this technique, the size of resulting vesicles is larger than that prepared by high shear condition. MLV and LUV are usually obtained by this method.

G. High pressure homogenization and microfluidization

This method based on the mechanical technique which will be used to generate vesicles in one step without organic solvents or lyophilazation requirement. It was introduced by Mayhew et al. in 1987. The liposomes are directly produced by high shear homogenizer treatment of the lipid-mixing powders. After equilibrium period, SUV will be obtained from this technique. The entrapment efficiency of vesicles prepared by this method depends on pressure and time (numbers of cycles) (Rodrı´guez and Sabe´s, 2001). High sheer homogenizers have also been used to reduce size of MLV and narrow down size distribution (Brandl et al., 1990).

H. Supercritical carbon dioxide fluid technique ($scCO_2$)

H.1 Characteristics

A supercritical fluid is a substance above its critical temperature (Tc) and critical pressure (Pc). Under these conditions, there is no distinction between gases and liquids and the substance can only be described as a

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Table 7 The critical temperature, pressure and density of substances common used as supercritical fluids (Reid et al., 1987)

Solvent	Critical temperature K (°C)	Critical Pressure MPa (atm)	Critical density (g/cm^3)
Carbon dioxide (CO_2)	304.1 (31.1)	7.38 (73.8)	0.469
Water (H_2O)	647.3 (374)	22.064 (217.755)	0.322
Methane (CH_4)	190.4 (-83)	4.60 (45.4)	0.162
Ethane (C_2H_6)	305.4 (32)	4.87 (48.1)	0.203
Propane (C_3H_8)	369.8 (97)	4.25 (41.9)	0.217
Ethylene (C_2H_4)	282.4 (9)	5.04 (49.7)	0.215
Propylene (C_3H_6)	364.9 (92)	4.60 (45.4)	0.232
Methanol (CH_3OH)	512.6 (239)	8.09 (79.8)	0.272
Ethanol (C_2H_5OH)	513.9 (241)	6.14 (60.6)	0.276
Acetone (C_3H_6O)	508.1 (235)	4.70 (46.4)	0.278

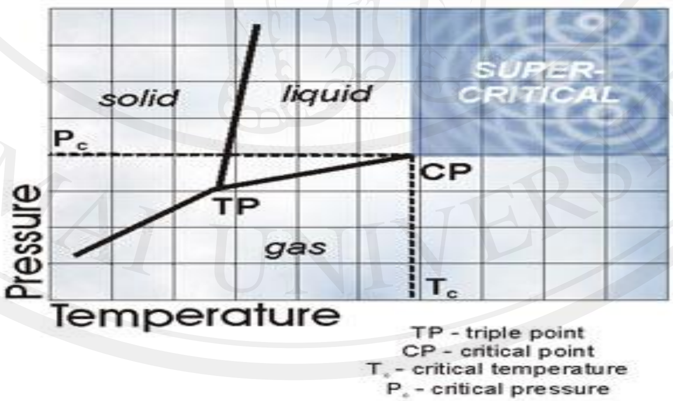

Figure 10 Pressure-temperature phase diagram and supercritical fluid region

fluid. The critical temperature, pressure and density of substances which are commonly used as supercritical fluids are shown in **Table 7** (Reid et al., 1987). One of the most important characteristics of supercritical fluids is solubility. At constant temperature, solubility in supercritical fluids tends to increase with density or pressure

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of the fluids. At constant density, their solubility will increase with temperature (Gorbaty and Bondarenko, 1998). **Figure 10** shows pressure-temperature phase diagram with the region of supercritical fluid. Carbon dioxide is the most widely used to prepare supercritical fluid due to low critical points (Tc = 31.1 °C and Pc = 73.8 bar), non-toxic, non-flammable and cheap. Supercritical carbon dioxide ($scCO_2$) is an excellent non-polar solvent for many organic compounds. It has been likened to a solvent resembling hexane. Alkenes, alkanes, aromatics, ketones and alcohols (up to a relative molecular mass of around 400) can dissolve in $scCO_2$, whereas very polar molecules such as sugars or amino acids and most inorganic salts are insoluble (Akgerman and Madras, 1994).

H.2 Applications

Supercritical fluid has been first demonstrated as a solvent in liquid chromatography by Klesper and co-workers in 1962. However, a major development of supercritical fluids is focused on particle formation in pharmaceutical, nutraceutical, cosmetic and specialty chemistry industries. Particle formations by supercritical fluid are categorized into four groups according to the role of supercritical fluid in the process as solvents, solutes, anti-solvents or reaction media (Reverchon and Adami, 2006). Several supercritical based particle formation techniques have been proposed by several studies as the followings:

(i) Rapid expansion of supercritical solutions (RESS)

This process consists of the saturation of the supercritical fluid with a solid substrate. Then, the depressurization of the solution through a heated nozzle into a low pressure chamber produces a rapid nucleation of the substrate in form of

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very small particles that are collected from the gaseous stream. The morphology of the resulting solid material (crystalline or amorphous) depends on the chemical structure of the material and the RESS parameters for examples temperature, pressure drop, distance of the jet against a surface and nozzle geometry (Jung and Perrut, 2001; Reverchon and Adami, 2006). This process is attractive due to the absence of organic solvent. However, its application is restricted to products that present a reasonable solubility in supercritical carbon dioxide (low polarity compounds). Sane et al. (2003) have used RESS to produce fluorinated tetraphenylporphyrin (a photosensitizer for photodynamic therapy) spherical, agglomerated nanoparticles with average particle sizes from 60 to 80 nm. Pathak et al. (2006) have reported that a nanosized ibuprofen (water-insoluble drug) particles in aqueous suspension can be formed using RESS. Manosroi et al. (2008) have prepared niosomes, non-ionic surfactant bilayer vesicles and enhance the entrapment efficiency of the water-soluble compound in niosomes by the modified RESS technique.

(ii) Gas or supercritical fluid anti-solvent (GAS or SAS)

This process is mostly used for recrystallization of solid dissolved in a solvent. A liquid solution contains the solute to be micronized. At the process conditions, the supercritical fluid should be completely miscible with the liquid solvent whereas, the solute should be insoluble in the supercritical fluid. Therefore, contacting the liquid solution with the supercritical fluid induces the formation of a solution, producing supersaturation and precipitation of the solute. The resulting particles can be either small size particles or large crystals, depending on the anti-solvent pressure variation rate. Tetracycline, an antibiotic, is an example of a

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pharmaceutical compound successfully processed by SAS. It has been obtained using *N*-methyl 2-pyrrolidone (NMP) as solvent. The mean particle size of precipitated particles is about 150 nm (Reverchon and Della Porta, 1999). Wu et al. (2005) have used SAS to micronize pigment Red 177 from DMSO. The spherical nanoparticles with mean diameter of 46 nm are obtained. Padrela et al. (2009) have produced indomethacin–saccharin cocrystals with different morphologies and sizes (nano-to-micron) using SAS. This work has demonstrated the potential of supercritical fluid technologies as screening methods for cocrystals with possibilities for particle engineering.

(iii) Solution enhanced dispersion by supercritical fluids (SEDS)

SEDS has been developed in 1994 by Hanna in order to achieve smaller droplet size. This process consists of a nozzle with two coaxial passages allowing to introduced the supercritical fluid and a solution of active substances into the particle formation vessel, where pressure and temperature are controlled. The high velocity of the supercritical fluid allows to break up the solution into very small droplets. Kang et al. (2008) have prepared poly(l-lactic acid)/ poly(lactide-co-glycolide) (PLLA/ PLGA) microparticles with the mean particle size of 1.76 to 2.15 µm. This study has indicated the potential of SEDS as an advanced colloidal suspension for pharmaceutical applications.

(iv) Particles from gas-saturated solutions (or suspensions) (PGSS)

PGSS is designed for making particles of materials that absorb supercritical fluids at high concentrations. Supercritical fluid is dissolved in a liquid solution of the substrates in a solvent, or a suspension of the substrates in a solvent followed by a rapid depressurization of this mixture through a nozzle causing the

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formation of solid particles or liquid droplets according to the system. Rodrigues et al. (2004) have used PGSS to encapsulate theophylline in hydrogenated palm oil for controlled drug delivery system. Jordan et al. (2009) have prepared sustained release formulations of hGH in PLGA/ PLA microparticles using PGSS technique. The results have indicated the high entrapment efficiency (100%) of protein in microparticles with no structural changes of the protein.

H.3 Advantages

As aforementioned, supercritical fluids tend to be applied as a solvent in several fields for examples pharmaceutical, nutraceutical, cosmetic and chemistry industries. This is due to their unique advantages over the conventional organic solvent (Ekart et al., 1991) as the followings:

(i) Dissolving characteristics can be varied by alteration of pressure and temperature

(ii) Low-energy solvent recovery due to lower evaporation temperature than conventional solvents

(iii) Physiologically harmless solvent

(iv) Non-inflammable solvent

(v) Gentle treatment of temperature-sensitive substances (for example natural substances)

(vi) Solvent-free and high-purity products

1.4.4.3 Physicochemical characteristics of nanovesicles

The effectiveness and stability of nanovesicles is strongly dependent on their physicochemical characteristics such as size, charge, lamellarity, surface properties and entrapment efficiency (Goren et al., 1990; Crommelin and Schreier,

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1994; Muller-Goymann, 2004). In order to ensure the reproducibility in clinical effects of vesicles, their characteristics are investigated immediately after preparation and during storage. The physicochemical parameters and techniques for characterization of vesicles have been demonstrated as the followings:

A. Vesicular size

The vesicular size is an important parameter in the in-process control and particularly in the quality assurance, because the physical stability of vesicle dispersions depends on vesicular size and size distribution. Some studies have proposed electron microscopy for size determination (Rose, 1980). However, the high cost-equipment and complicated data evaluation of electron microscopy make it less favorable as a routine technique. The regular technique to follow size stability of vesicle is dynamic light scattering (DLS) or photon correlation spectroscopy (PCS). This technique can be applied for diameter between a few nm and a few µm. However, the results are precise and accurate for only homogeneous dispersions (Ruf et al., 1989). Van Hoogevest and Frankhauser (1989) have suggested the coulter counter technique for size determination of heterogeneous vesicular dispersions in the size range of supra-µm. For heterogeneous vesicular dispersions in the nm range, their mean size and size distribution can be obtained by the exclusion chromatography (Dos Ramos and Silebi, 1990). Zhu et al. (2005) have investigated the stability of lactosyl liposomes (surface modified liposome) by examination of vesicular size using photon correlation spectroscopy. The results show that lactosyl liposomes have a narrow size distribution and remain stable at room temperature for at least one month.

B. Morphology

Morphology of vesicle has been proposed as a tool for

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understanding the formation, stability and entrapment efficiency of the vesicles (El Maghraby et al., 2000b; Zasadzinski et al., 2001; Waninge et al., 2003; Abreu et al., 2007). Several techniques can be used for the morphology determination such as electron microscope and small angle x-ray scattering (SAXs). Manosroi et al. (2005) have characterized the morphology of vesicles entrapped with kojic acid by the Bangham method with sonication using transmission electron microscope (TEM). The stable oligolamellar vesicles could be formed in this study. Fernandez et al. (2008) have used SAXS to determine the interbilayer distances of DMPG vesicles in the presence of sodium salts. The results have indicated that loose multilamellar structure start to appear with 50 mM of NaCl.

C. Charges

The charge density on vesicles can be estimated from mobility measurements in an electrical field (microelectrophoresis). The mobility data can be converted into zeta potential and then calculated to charge density using dynamic light scattering (Grit and Crommelin, 1993). Charges inducing agent for examples phosphatidyl glycerol (PG), phosphatidic acid (PA) or phosphatidyl serine (PS) is regularly added to bilayer to improve the physical stability against aggregation or fusion, and also increase the biological activity (Leonards, 1998; Arouri et al., 2009). Manosroi et al. (2008) have reported that the stability of luciferase plasmid can be enhanced by entrapping in cationic liposomes.

E. Entrapment efficiency

The entrapment efficiency is a crucial parameter in the characterization of the vesicles. This parameter gives information on the level of

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excepient that should be administered at each dose level. For corrected entrapment efficiency determination, the unentrapped excepient has to be removed. Several techniques have been used for the removal of the unentrapped excepient including exhaustive dialysis (Mokhtara et al., 2008), gel filtration (Manosroi et al., 2005), centrifugation (Yang, 2007) and ultracentrifugation (Brgles et al., 2009). The advantages and disadvantages of these methods are shown in **Table 8**.

1.4.5 Biological assays

1.4.5.1*In vivo* skin irritation assay in rabbits

In cosmetic industry, evaluation of irritancy potential to human skin of any chemicals or formulations is necessary. This must be done by the means of *in vivo* and *in vitro* tests to determine the risk of irritation due to the contact between these compounds and human skin. The most commonly used test is the rabbit skin irritation test described in the OECD test guideline 404 and in the European Chemicals Bureau Annex V part B.4 (http://ecb.jrc.it/testing-methods/) which was initially described by Draize et al., (1944). In this animal test, the test substances, either raw materials or finished formulated products, are applied on the rabbit's shaved skin. A score of skin reactions is based on the physiological observations on the animals. After the tested products are applied on the rabbit skin, the rabbits are examined for the presence of erythema and oedema according to the Draize dermal irritation scoring system (0: no erythema or no oedema; 1: barely perceptible erythema or oedema; 2: well defined erythema or slight oedema; 3: moderate to severe erythema or moderate oedema; 4: severe erythema or oedema) at the grading intervals of 1, 24, 48 and 72 h. The skin irritation potential of the chemicals is often summarized as the 'primary irritation index' (PII) calculated from the erythema and oedema grades according to the

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Table 8 The advantages and disadvantages of different methods for the separation of the vesicles from the unentrapped drug (Uchegbu and Vyas, 1998)

Method	Advantages	Disadvantages
Exhaustive dialysis	- Suitable for large vesicles\ 10 mm - Suitable for highly viscous systems - Inexpensive	- Extremely slow (5-24 hr) - Large volumes of dialysate required-(may not be suitable for drugs requiring specialised disposal) - Dilutes the niosome dispersion
Centrifugation (below 7000xg)	- Quick (~30 min) - Concentrates the niosome dispersion - Inexpensive	- Fails to sediment the sub-micron niosomes - May lead to the destruction of fragile systems
	Instrumentation	
Ultracentrifugation (150000xg)	- Sediments all size populations - Concentrates the niosome dispersion	- Expensive Instrumentation - Long centrifugation times (1-1.5 hr) - May lead to the destruction of fragile systems - May lead to the formation of aggregates

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following formula: PII = [($\sum$ *erythema grade at 24/48/72 h* + $\sum$ *edema grade at 24/48/72 h)/ 3* $\times$ *number of animals*].

The irritation degree was categorized based on the PII values as negligible (PII = 0–0.4), or slight (PII = 0.5–1.9), moderate (PII = 2–4.9) or severe (PII = 5–8) irritation.

1.4.5.2 *In vitro* assays for cosmetic uses

A. DPPH free radical scavenging activity assay

The free radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydracyl), a stable free radical, has been widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) or hydrogen donating activities of various compounds since it is a simple, rapid and sensitive method (Suja et al., 2005; Letelier et al., 2008). DPPH, a radical generating substance, has a deep violet color due to its unpaired electron. Free radical scavenging ability can be followed by the loss of the absorbance at 515 nm as the pale yellow non-radical form is produced. After DPPH solution react with the samples, the absorbance of the resulting solutions are measured and compared with the absorbance of DPPH in the absence of sample solution. Lower absorbance represents higher activity. The reaction of the DPPH radical in the presence of the antioxidant compound during the DPPH assay is shown in **Figure 11**.

B. 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 & Shahidi, 2007). Fe^{2+}-ferrozine complex method is commonly used for the evaluation of chelating properties of the compounds. In the presence of chelating agents, the complex

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Deep violet color at 515 nm

Pale yellow (non-radical form)

Figure 11 Reaction of the DPPH radical in the presence of the antioxidant during the DPPH assay (Prakash, 2001)

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).

C. 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 12**). 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,

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copper-containing protein, is a key enzyme for melanogenesis. Tyrosinase existing in the skin can catalyze the oxidation of L-tyrosine to L-DOPA and subsequently to L-DOPA quinone which will mediately change to melanin, a brown to black color pigment. Therefore, any compounds which can inhibit this enzyme, can inhibit the formation of melanin.

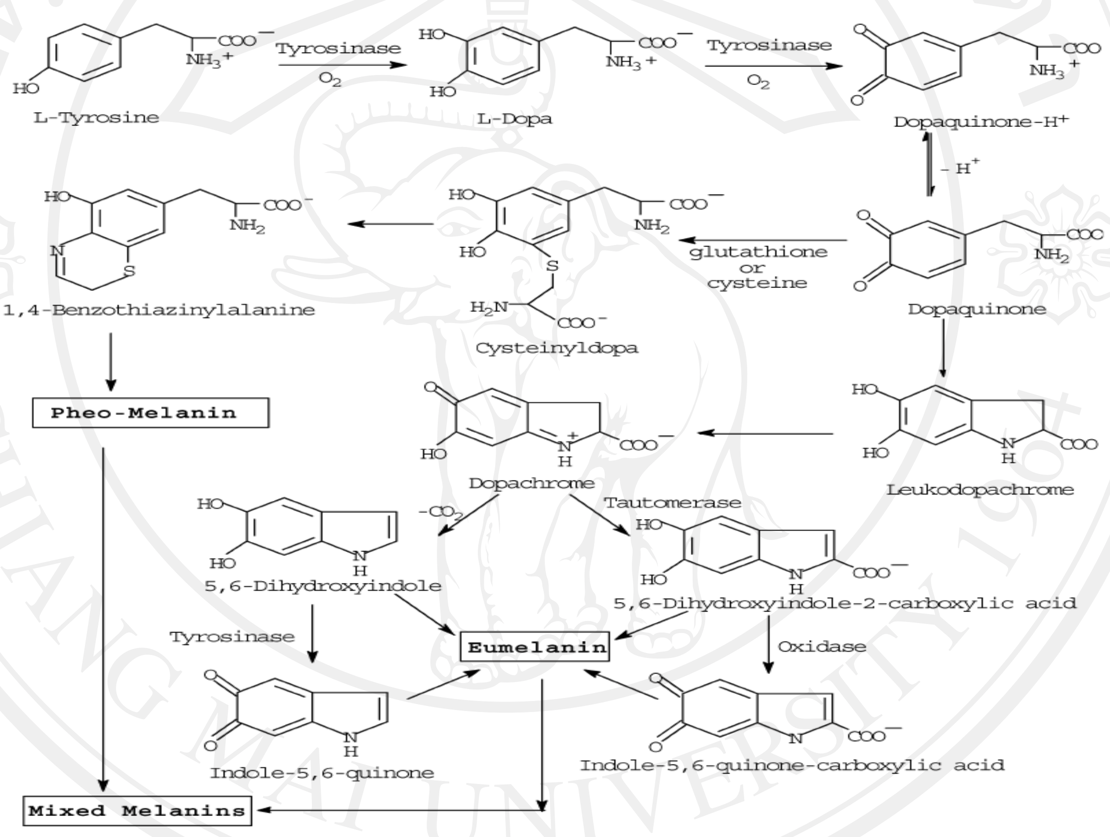

Figure 12 Melanogenesis pathway (Sung-Yum et al., 2003)

D. Lipid peroxidation inhibition assay

The polyunsaturated fatty acids, for examples, linoleic acid are mainly presented in the biological membranes. They can be oxidized by unspecific reactive oxygen species (ROS) known as lipid peroxidation (LPO). This process is a radical mediated pathway and a highly damaging event including atherosclerosis, cataract, rheumatoid arthritis, and neurodegenerative disorders (Niki,

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2005). Thus, the evaluation of the antioxidants ability to counteract with lipid peroxidation has been extensively studied (Laguerre, 2007).

Ferric thiocanate method in linoleic system has been widely used to evaluate LPO inhibition activity of the compounds. In this assay, the solution of ferrous chloride ($FeCl_2$) and ammonium thiocyanate (NH_4SCN) react with each other to produce ferrous thiocyanate (red color) by the means of hydroperoxide, which is generated by linoleic acid oxidation during the experimental period. The absorbance of the reacted solution is measured at 500 nm. The lower absorbance represents the higher activity (Suja et al., 2005). The reaction of lipid peroxidation in ferric thiocyanate assay is shown in **Figure 13**.

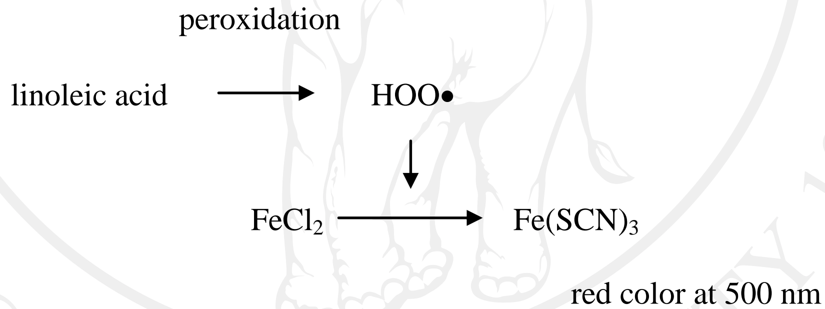

Figure 13 The reaction of lipid peroxidation in ferric thiocyanate assay

E. Cell proliferation activity

In vitro cell proliferation assay in cancer cell has been recognized as a primary tool for the screening of anticancer agents. Mosmann (1983) established the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and subsequently, modified tetrazolium salts like XTT and WST-1 have become available. Using these assays, a large number of tests can be carried out in a rapid, reproducible and sensitive fashion. The development of the sulforhodamine

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B (SRB) protein binding assay for the *in vitro* measurement of cellular protein content of the adherent and suspension cultures for the evaluation of cytotoxicity and cell proliferation in microplate was established by Skehan et al. (1990). Subsequently, it was adopted for routine use in the National Cancer Institute (NCI, USA) *in vitro* antitumor screening.

MTT

Formazan

Figure 14 Structure of MTT and their corresponding reaction products (Mosmann, 1983)

E.1 MTT assay

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

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formazan crystal can be solubilized and quantified by a multiwell spectrophotometer (microplate reader). The structure of MTT and their corresponding reaction products are shown in **Figure 14**.

E.2 SRB assay

SRB is a bright pink aminoxanthene dye. It posseses 2 charged SO_3^- groups which are capable of electrostatically binding to positive counter ions. Under mildly acidic conditions, SRB binds to the positive fixed charges of biological molecules. In TCA fixed cells, these binding sites are primarily the amino groups of the proteins. The SRB binds to the basic amino acids of cellular macromolecules and the colorimetric evaluation provides an estimation of total protein mass which is related to cell growth or viability in the treated and untreated cells. SRB behaves like a bromphenol blue, naphthol yellow S, and Coomassie Blue, which are also used widely as protein stains. With TCA fixed cultures, SRB gives a higher OD and better signal to noise ratio at low cell density than do these other dyes. The method has replaced the tetrazolium based assays by exhibiting a number of advantages including better linearity, higher sensitivity, a stable end point that does not require time-sensitive measurement and lower cost and toxicity (Papazisis et al., 1997).

F. Gelatinolytic activity on MMP-2 inhibition (Zymography)

F1. Types of collagen and MMPs

Collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. More than 90% of the extracellular protein in the tendon and bone and more than 50% in the skin consist of collagen (Piez, 1985). Different collagen types are necessary to confer distinct

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biological features to various types of connective tissues in the body. Collagen comprises a family of genetically distinct molecules which have a unique triple-helix configuration of three polypeptide subunits known as α-chains in common. Currently, at least 13 types have been isolated which vary in the length of the helix and the nature and size of the non-helical portions (**Table 9**) (Kucharz, 1992).

MMPs are a family of 28 proteolytic endopeptidases (**Table 10**) containing a Zn (II) ion held in the active site by three histidines (Sternlicht & Werb, 2001). This family is called matrix metalloproteinases because of their ability to degrade the extracellular matrix (ECM). Their dependence on a metal ion (Zn II) for their activity and their sequence and domain similarities distinguish them from other closely related metalloprotinases. MMPs are primarily involved in tissue remodeling, but are also implicated in cell-signaling by releasing of growth factors, aging, embryogenesis, angiogenesis, endometrial maintenance and bone remodeling (Schulz, 2007). As well as cancer, MMPs are also implicated in a number of diseases like rheumatoid arthritis, periodontal disease and intestinal inflammation (Jacobsen et al, 2007; Schulz, 2007).

F2. Collagen and MMPs in aging

Changes in the production and formation of collagen and elastic fibers are common characteristics of an aging dermis (El-Domyati et al., 2002). The relative proportion of the types of collagen in skin also changed with age. Young skin is composed of approximately 80% of type I collagen and about 15% of collagen type III. In an aged skin, collagen fibers became thicker and there was a loss of collagen type I, which altered the ratio of the collagen types (Oikarinen, 1990). Moreover, aged fibroblasts synthesized lower levels of collagen, both *in vitro* and *in vivo*, compared to young adult fibroblasts (Varani et al., 2006). Fisher et al. (2009) reported that dermal

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Table 9 Body distribution of collagen types (Kucharz, 1992)

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

Table 10 Types of MMPs (Snoek-van Beurden & Von den Hoff, 2005)

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

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fibroblasts express higher levels of MMP-1 in aged human skin *in vivo*, when compared with fibroblasts from young skin. In addition, the annual loss of total collagen was approximately 1% from 50 years of age. Senescent fibroblasts exhibit phenotypic changes, becoming wide and flat with irregular shapes and lobulated nuclei (Kletsas, 2003). In an aged skin, there is an accumulation of elastosis and further degradation of the extracellular matrix due to the action of MMPs, which decreased collagen levels (Yin et al., 2000). MMPs (collagenase, gelatinase and stromelysin) can degrade collagen fibers, although collagen is relatively stable. Interstitial collagenase (MMP-1) produced by interstitial fibroblasts and inflammatory cells (macrophages and leukocytes) can degrade collagen types I and III. MMP-1 introduces a single break in each molecule of the triple α-chain, thus denaturing the collagen molecule. Metalloproteinases-9 (MMP-9) and metalloproteinases-2 (MMP-2) can degrade collagen IV (Gross & Lapiere, 1962). After the initial proteolytic cleavage, collagen can be degraded by other proteolytic enzymes (Horwitz et al., 1977; McCroskery et al., 1975). In general, this process is similar for all types of collagenase. However, differences exist in the susceptibilities of different types of collagen to various collagenases. For example, MMP-1 cleaves type I collagen more rapidly than it cleaves type III collagen (Horwitz et al., 1977).

F3. Zymography

Zymography is an electrophoretic technique based on SDS-PAGE that includes a substrate co-polymerized with the polyacrylamide gel, for the detection of enzyme activity (Lantz &Ciborowski, 1994). Samples are prepared in the standard SDS-PAGE treatment buffer but without boiling, and without a reducing

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agent. Following electrophoresis, the SDS is removed from the gel (or zymogram) by incubation in unbuffered Triton X-100, followed by incubation in an appropriate digestion buffer, for an optimized length of time at 37°C. The zymogram is subsequently stained (commonly with Amido Black or Coomassie Brilliant Blue), and the areas of digestion appear as clear bands against a darkly stained background where the substrate has been degraded by the enzyme (Snoek-van Beurden & Von den Hoff, 2005). Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9 (**Figure 15**). It is extremely sensitive because levels of 10 pg of MMP-2 can already be detected (Kleiner & Stetler-Stevenson, 1994). It should be considered, however, that other MMPs, such as MMP-1, MMP-8, and MMP-13 can also lyse the substrate. This signal will probably be very weak because gelatin is not their preferential substrate. For MMPs that do not show any activity on gelatin, modifications of the technique have been made for an improved detection. This is mainly done by incorporating a more suitable substrate into the gel, such as casein or collagen, or by enhancing the signal by adding heparin to the samples (Gogly et al., 1998; Yu & Woessner, 2001).

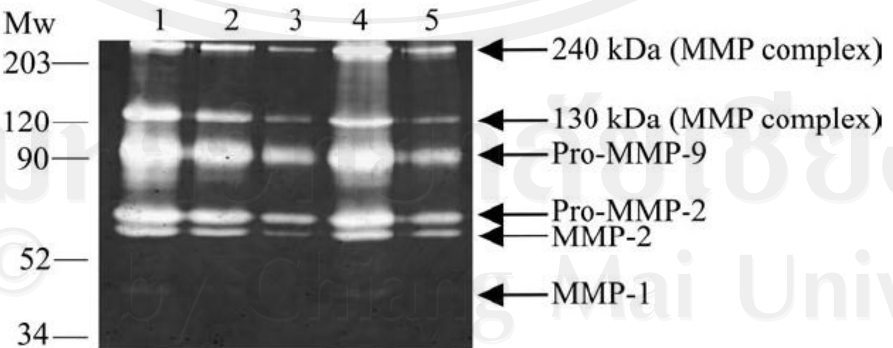

Figure 15 Gelatin zymography (Snoek-van Beurden & Von den Hoff, 2005)

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