Skin Evaluation of Creams Containing *Phyllanthus emblica* Fruit Extract Liposomes

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ชื่อวิทยานิพนธ์	การประเมินประสิทธิภาพทางผิวหนังของครีมที่มีลิโปโซมจากสารสกัดมะขามป้อม
ผู้เขียน	นายปณิธิ รักนาม
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ปีการศึกษา	2554

บทคัดย่อ

Phyllanthus emblica Linn. หรือมะขามป้อม เป็นพืชในวงศ์ Euphorbiaceae ซึ่งมีประวัติการใช้ประโยชน์จากมะขามป้อมระยะเวลายาวนานทั้งเพื่อบริโภค และเพื่อการรักษาทางการแพทย์ ผลมะขามป้อมยังอุดมไปด้วยวิตามินซี ธาตุอาหารต่างๆ รวมทั้งสารในกลุ่มฟีนอลิก และ แทนนิน ซึ่งสารเหล่านี้มีฤทธิ์ในการต้านอนุมูลอิสระสูง ฉะนั้นมีความเป็นไปได้ในการพัฒนาเป็นผลิตภัณฑ์ทางเครื่องสำอาง แต่อย่างไรก็ตามสารออกฤทธิ์ดังกล่าวในผลมะขามป้ คุณสมบัติเป็นกรด และยังมีสภาพขั้วสูงอาจจะทำให้ระคายเคืองต่อผิวหนัง รวมทั้งมีข้อจำกัดบางประการในแง่ของการซึมผ่านทางผิวหนัง ลิโปโซมเป็นระบบนำส่งทางผิวหนังที่มีประสิทธิภ และเป็นที่นิยมในทางเครื่องสำอาง คณะผู้วิจัยจึงทำการศึกษาผลทางคลินิกเพื่อเป็นการพิสูจ ศักยภาพของผลิตภัณฑ์

จากการศึกษาฤทธิ์ต้านอนุมูลอิสระของผลมะขามป้อมด้วยวิ DPPH radical scavenging assay พบว่ามะขามป้อมมีค่าความเข้มข้นที่สามารถกำจัดอนุมูลอิสระไ 50% (EC_{50}) เท่ากับ 2.48 ± 0.03 ไมโครกรัมต่อมิลลิลิตร สารสกัดมีปริมาณสารประกอบฟีนอลิกทั้งหมด 274.58 ± 0.007 มิลลิกรัมต่อกรัมของสารสกัดเทียบกับกรดแกลลิค โดยการทดลองครั้งนี้ใช้กรดแกลลิคใช้เป็นสารมาตรฐานในการประเมินด้านคุณภาพ การศึกษาที่ผ่านมาได้มีการพัฒน

ลิโปโซมมะขามป้อมได้สูตรที่ดีที่สุดคือ SPC: Tween: DA โดยใช้ปริมาณไขมันรวม 200 ไมโครโมลต่อมิลลิลิตร โดยมีขนาดอนุภาคเท่ากับ 247.7 ± 5.49 นาโนเมตร และประสิทธิภาพการเก็บกักสารสำคัญเท่ากับ 46.79 ± 0.21 เปอร์เซ็นต์ ที่ความเข้มข้นของมะขามป้อม 1 เปอร์เซ็นต์ (โดยน้ำหนัก) การพัฒนาสูตรตำรับเครื่องสำอางโดยมีลิโปโซมมะขามป้อมในตำรับอิมัลชันชนิดน้ำมันในน้ำ และพบว่าสูตรตำรับมีความคงตัวทางเคมีกายภาพ อีกทั้งยังสามารถซึมผ่านผิวหนังได้ดี จากนั้นจึงนำไปทดสอบกับอาสาสมัครจำนวน 40 ราย ช่วงอายุ 25 ถึง 45 ปีโดยแบ่งการทดลองออกเป็น 2 กลุ่ม คือกลุ่มที่ใช้ครีมลิโปโซมมะขามป้อม และกลุ่มควบคุม (ครีมลิโปโซม) โดยระยะเวลาทดสอบ 1 เดือน อาสาสมัครจะต้องมาทำการประเมินสภาพผิวในสัปดาห์ที่ 1, 2 และ 4 จากผลการทดลองพบว่า อาสาสมัครกลุ่มที่ใช้ครีมลิโปโซมมะขามป้อมมีผิวสว่างกระจ่างใสขึ้นหลังจากใช้เป็นเวลา 2 สัปดาห์ และในสัปดาห์สุดท้ายอาสาสมัครมีผิวขาวขึ้น 30 เปอร์เซ็นต์เทียบกับเมื่อใช้ผลิตภัณฑ์ นอกจากนี้พบว่าความยืดหยุ่นของผิวมีค่าเพิ่มสูงขึ้น และปริมาณจุดด่างดำจางลงอย่างมีนัยสำคัญทางสถิติ ผลการทดสอบการประเมินความพึงพอใจของผู้ใช้ผลิตภัณฑ์พบว่า อาสาสมัครพึงพอใจประสิทธิภาพของครีมลิโปโซมมะขามป้อม และจากผลการประเมินประเมินประสิทธิภาพทางคลินิกแล้วแสดงให้เห็นว่าครีมลิโปโซมมะขามป้อมมีศักยภาพในการเป็นผลิตภัณฑ์เครื่องสำอางเพื่อผิวขาวได้ดี

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ABSTRACT

Phyllanthus emblica Linn. (synonym: *Emblica officinalis* Gaertn.) also known as Ma-kham-pom in Thai, has been traditionally used both in consumptions and medicinal treatments for a long time. It is an importance dietary source of vitamin C, minerals and also contains phenolic compounds and tannins. According to chemical constituents in emblica extract that possess antioxidant activities are acidic properties and also high polarity that might be a problem in terms of skin irritation and skin permeation. Liposomes are one of many delivery systems to skin that popularly used in cosmetics for effective skin permeation. Then the study of liposomes cream of this extract that affect on facial skin of volunteer was determined to prove a potential of product.

In this study, the free radical scavenging of emblica extract was found 2.48 ± 0.03 µg/mL in term of EC_{50} and total phenolic content was 274.58 ± 0.007 mg/g GAE. Based on HPLC analysis, gallic acid was found as constituent of the extract. Gallic acid, therefore, used as indicative marker in the quality control of the extract using HPLC technique. The result was found that the content of gallic acid in the extract was 34.27 ± 0.67 mg/g. The results obtained from this study indicated that

emblica fruit has been shown to be a safe and effective to develop as the cosmetic products. Thus the emblica liposomes were successfully prepared comprising SPC (phosphatidylcholine from soya), Tween 80 and Deoxycholic acid at the ratio 84: 16: 2.5 (weight ratio) as well with the total lipid 200 μmol/mL. Furthermore, the size of liposomes was 247.7 ± 5.49 nm and entrapment efficiency was 46.79 ± 0.21% of 1% w/w of emblica extract liposomes. The possibility of formulation such emblica liposomes in an o/w emulsion were determined for good physicochemical properties and also good skin permeation. After that a study to evaluate the clinical efficacy and safety of emblica extract combined with liposomes as active transport in human volunteers was examined. A randomized double blind placebo controlled study was carried out for 4 weeks on 40 volunteers aged between 25 to 45 years. The subjects were divided in 2 groups; the first group received the 1% emblica liposomes cream, whereas the control received the 1% emblica liposomes cream as control. The subjects in each group applied the formulations twice a day. All subjects were evaluated in 4-week study; week 0, week 1, week 2 and week 4, they were submitted to cosmetic evaluation. The emblica extract was effective, giving the short onset significant whitening effect after use only 2 weeks. Skin transparency showed an important influence after use, good efficacy could be obtained for 80% in the subjects. Finally, the results obtained from this study might show that this product has a potential for whitening skin in cosmetic products.

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LIST OF ABBREVIATION AND SYMBOLS

Ab	absorbance
-	alpha-
AOAC	Association of Official Analysis Chemists
! -	beta-
BHA	butyl hydroxyl anisole
BHT	butyl hydroxyl toluene
^{o}C	degree Celsius
CHOL	cholesterol from lanolin
CIE	Commission International dé l Eclaisage
cm	centimeter
DA	deoxycholic acid
DPPH	2,2-diphenyl-1-pycryl-hydrazyl
EA	ellagic acid
EC_{50}	effective concentration of sample that scavenges DPPH radical by 50%
EE	encapsulation efficiency
g	gram
GA	gallic acid
GAE	gallic acid equivalent
HPLC	high-performance liquid chromatography
HPLC-DAD	high-performance liquid chromatography diode area detector
H_3PO_4	phosphoric acid
L	liter

LIST OF ABBREVIATION AND SYMBOLS (Continued)

LMV	large multilamellar vesicles
LPO	lipid peroxidation
LUV	large unilamellar vesicle
m	meter
#g	microgram
#L	microliter
#m	micrometer
M	molar
mg	milligram
min	minute
mL	milliliter
MLV	multilamellar vesicle
mm	millimeter
MVV	multivesicular vesicle
o/w	oil-in-water
NaCl	sodium chloride
MAP	magnesium ascorbyl phosphate
nm	nanometer
Pa	pascal
PC	phosphatidylcholine
PCS	photon correlation spectrometer
r^2	correlation coefficient
REV	reverse phase evaporation

LIST OF ABBREVIATION AND SYMBOLS (Continued)

rpm	round per minute
ROS	reactive oxygen species
RSD	relative standard deviation
SA	stearylamine
SD	standard deviation
SOD	superoxide dismutase
SPC	3-sn-phosphatidylcholine from soy bean
SUV	small unilamellar vesicle
TEWL	transepidermal water loss
UV	ultraviolet
UVA	ultraviolet A
UVB	ultraviolet B
UVC	ultraviolet C
v	volume
w/o	water-in-oil
w	weight
w/w	weight-by-weight

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Personal appearance is a good way to generate interest especially for women. The well-being and physical characteristics are largely influenced by person's opinion. Today, cosmetics are one of the most important growing businesses in the world as results of the wide variety consumption of cosmetic products (Patravale and Mandawgade, 2008). The basic concepts of cosmetic products are emphasized in order to improve and encourage physical appearances. Because of focusing on their health, consumers worldwide explore a renewed interest in the use of naturally occurring compounds (Kapoor *et al*., 2009). In the periodical currently of natural antioxidant era, this trend has been increased considerably in finding newish natural antioxidants for use in pharmaceutical and cosmetic products to replace the synthetic substances. Since the natural antioxidants are originated from natural source, low toxicity and safety point of view (Morganty, 2009; Fuller, 2010).

The use of natural antioxidants in cosmetic products has a long period of time. Antioxidant activity is an excellent example of a functional benefit that plant extract can deliver. Plants are known to contain a variety of natural antioxidants that protect and preserve their physical and metabolic integrity as well. Currently antioxidants are widely incorporated into a variety of antiageing skin care systems.

The commonly used cosmetic antioxidant ingredients include vitamins A, B, C, E, CoQ_{10} its analogues. Some of these compounds can be extracted and purified from plants. Certainly it is possible to be reasonable strategy to support the natural defense system of the skin by application of antioxidants. Comprising a board range of naturally compounds, polyphenols are secondary plant metabolites that exert varying degrees of antioxidant activity (Baumann, 2009). Plant phenols and polyphenols constitute another large and important group of naturally occurring antioxidants by virtue of the fact that the phenolic group can stabilize oxidative radicals. They are widely dispersed throughout the plant kingdom. Flavonoids, flavonoid glycosides, catechins, proanthocyanidins, flavonolignans and phenylpropanoids are all examples of plant phenolic compounds for which there is much supporting evidence for antioxidant benefit to skin. Plant phenolic compounds have shown direct antioxidant in quenching superoxide anion, oxidative radicals and lipid peroxidation. Many member of this group have also possessed anti-inflammatory and anti-microbial properties (Angerhofer *et al*., 2008). Then, many medicinal plants are available as ingredients in the market today. A board spectrum of medicinal plants has been reported for their antioxidant properties, for instance, *Hibiscus sabdariffa* Linn., *Curcuma longa*, *Aloe barbadensis* and *Punica granatum* Linn.

Indian gooseberry, also names as emblica (*Phyllanthus emblica* Linn.) fruits are widely consumed fresh and in commercial products as juices, jam and used in many traditional medicine systems herbal drug in form of Triphla which is a formulation containing three fruits of *P. emblica*, *Terminalia chebula* Rotz. and *Terminalia bellerica* Roxb. in equal proportions (Khan, 2009). Its fruit juice contains the highest dietary source of vitamin C which blended with other fruits, boosted their

nutritional quality in terms of vitamin C content (Chakraborty and Verma, 2010). Traditionally, the fruit is useful astringent, cardiac tonic, diuretic, laxative, livertonic, refrigerant, hair tonic and digestive medicine. It is reported to be effective in the treatment of peptic ulcer and dyspepsia. It is reported to have hepatoprotective, antioxidant, antimutagenic, cytoprotective, antitumor, antifungal, antimicrobial, hypolipidemic and antiantherosclerotic effects. Emblicanin is a type of antioxidant found in *P. emblica*. Emblicanin is different from most other antioxidants in that it is cascading antioxidant that is completely free of pro-oxidation (Ghosal *et al.*, 1996; Bhattacharya *et al*., 1999; Scartezzini *et al*., 2000; Zhang *et al*., 2001; Khan *et al*., 2002; Anila and Vijayalakshmi, 2003). Moreover, emblicanin seeks and attacks free radicals so it makes one of the best free radicals scavenging antioxidants. Emblica extracts are also being investigated for their potential use as the food biopreservatives and nutraceuticals. For this reasons, it is possible to assume that emblica extract can be promised as a good natural antioxidant and able to develop as an ingredient in cosmetic products especially for antiageing and skin whitening (Baumann and Allemann, 2009). As a matter of fact the chemicals that possess antioxidant activity in emblica are acidic compounds according to its structure that might irritate to the skin and also have a limit of skin permeation. So in this concept, the advance technology such as liposomes is useful for troubleshooting.

The clinical efficacy of emblica fruit is held in high esteem in Ayurveda and folk medicines. Emblica extract alone or as part of many polyherbal cosmetic formulations, currently are in use for skin related beauty care. Although there are many examples of application of liposomal formulations according to published articles but there is no reports on enhancement of emblica extract efficacy

by liposomes. From this point of view, it is possible to perform clinical study of cosmetic dosage forms containing emblica liposomes in human volunteers and to evaluate the effect of emblica extract.

1.2 The purpose of this study

The overall aims of this study were:

1.2.1 Develop cosmetic creams containing the emblica liposomes and evaluate their physical stability.

1.2.2 Evaluate the emblica liposomes creams by the *in vitro* skin permeation study as antioxidant activity indicator.

1.2.3 Evaluate the effects of the emblica liposomes creams on human skin by the *in vivo* study.

CHAPTER 2

LITERATURE REVIEW

2.1 Basic concepts in cosmetic

Nanotechnology is one of the key technologies of the 21st century which wide opens new perspectives for development science-based solutions for innovative application (Guimaraes and Re, 2011). One of these promising applications candidates to develop of refined cosmetic products whose final performance may be directly impacted by the characteristic nanoscale, thus representing excellent opportunities for both research and business (Jentzsch and Aikens, 2005; Trierweiler and Trierweiler, 2011).

Moreover, nanotechnology plays influent role surrounding us which are utilized largely in daily life. In the same way, advanced technology in cosmetic is not only superior but also progressive to comprehend innovations (Wilmott *et al*., 2005). To achieve in cosmetology, for this reason; it is necessary to study on the basis of skin, dosage forms, the use of integrated technology and final step the clinical study in human volunteers. The clinical study can be claimed that the cosmetic formulations are also believable in efficiency based on the published documents using modern scientific approaches. According to definition of cosmetic products are defined as articles intended to be rubbed, poured, sprinkled or sprayed on, introduced into, or otherwise applied to the human body or any part for cleansing, beautifying,

promoting attractiveness, or altering the appearances. The aim of beautifying products is to impact a pleasant and attractive appearance by emphasizing those areas of the face or body that look better, in order to focus the observer's gaze on them.

2.2 Skin structure pertinent to cosmetic

Based on human's body, skin is the largest organ and exposed to a board spectrum variety of biological, chemical and physical attacks (Saija *et al*., 2000; Roberts and Cross, 2002; Walters and Brain, 2004; Azzi *et al*., 2005; Birchall, 2006; Walters and Roberts, 2007; Murthy and Shivakumar, 2010; Contri *et al*., 2011) composed of two layers: epidermis and dermis (Mollet and Grubenmann, 2001; Shai *et al*., 2009). Underneath the dermis lies subcutaneous, this consists mainly of fat cells. Mainly layers of human skin consist of three layers whereas cosmetic dermatology fundamentally interest especially focused on epidermis as the skin barrier which forms the epidermis permeability barrier that prevents the loss of water and electrolytes (de Jager *et al*., 2006; Pillai *et al*., 2010). A major obstacle to skin permeation is the low penetration of drugs through the skin (Trotta *et al*., 2002).

2.2.1 Epidermis

Epidermis forms the outer layer, the main barrier properties of human skin (Barrett, 1969; du Plessis *et al*., 1992; du Plessis *et al*., 1994; Peck and Higuchi, 1997; Hadgraft, 1999; Bos and Meinardi, 2000; Verma *et al*., 2003; Davis *et al*., 2007; Rerek and Moore, 2007; Wickett *et al*., 2007; Miteva and Fluhr, 2008; Förster *et al*., 2009). At the base of this layer, the cells continuously divide to form new cells (Pillai *et al*., 2010). In fact, cells are made; they are pushed toward the surface by newly cells underneath them, eventually contact the keratinous layer. Then the

outermost cells, stratum corneum in the keratinous layer are eliminated. Beside, in epidermis layer composed of keratinocytes differentiate to form four layers, stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The epidermis that prevents water loss is generally thin. It is particularly in the skin of the eyelid; approximately 0.1 mm (Rhein and Babajanyan, 2007). The stratum corneum provides the principle barrier of skin permeation of topically applied in a lipid matrix (Dayan, 2006). This lipid matrix is composed of highly organized bilayers, containing mainly free fatty acids, ceramides, cholesterol and cholesterol sulfate (Flynn, 2002).

2.2.2 Dermis

Dermis lies between epidermis and subcutaneous fat. In human, the whole mass of the dermis may constitute 15-20% of total body. The functional of dermis is mainly made up collagen and elastin fibers also supporting the skin and its annexes (hairs and nails) (Celleno and Tamburi, 2009). Moreover, it contains blood vessels, nerves, sensory organs, sebaceous glands, sweat glands and hair follicles. Dermis provides support for the epidermis and also plays a role in regulating temperature, pressure and pain (Walters and Brain, 2004).

2.2.3 Subcutaneous (Hypodermis)

This layer also contains adipose tissue and serves to attach the dermis to its underlying tissues.

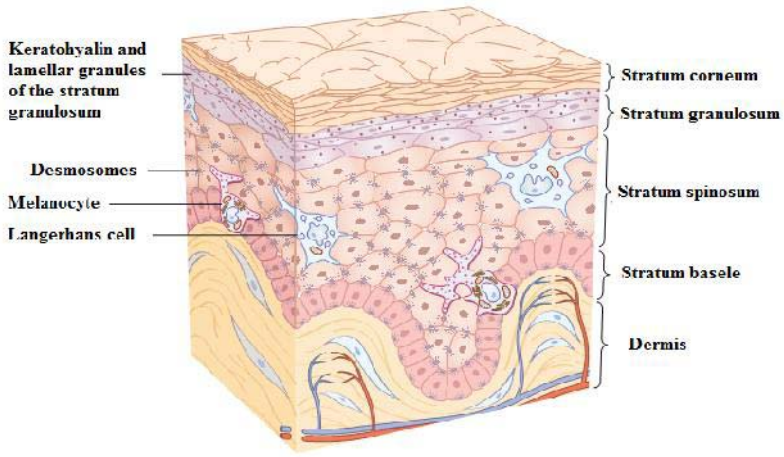


Figure 1 Layers of the epidermis and other structural components (Pillai *et al*., 2010)

2.3 Ageing and its solving

Skin, like all other organs, ages over time. Ageing, an inevitable biological process that affects most living organisms (Kaur *et al*., 2007) is associated with an age-related increasing in susceptibility to diseases and death (Ames *et al*., 1993; Harman, 1998; Schoneich, 1999; Barja, 2004; Wilson, 2008). Ageing can be seen by the naked eye which is deleterious, progressive, intrinsic, and universal. In the same way, ageing is a complicated and heterogeneous phenomenon (Taylor, 2005). In fact skin is the major candidate and target of oxidative stress. Based on the theory, ageing can be divided into two categories: intrinsic or chronological ageing and extrinsic also called premature or photo-ageing (McCullough and Kelly, 2005; Wilson, 2008). In short, intrinsic ageing is assorted with other physiological processes with numerous simultaneous mechanisms occur (Wilson, 2008). Collagen and elastin production slow down which fibers that make up the dermal matrix and give skin bounce back into its original position (Burlando *et al*., 2010). As older the skin capability to spring back to place is diminished. Skin becomes thinner, the dermal

epidermal junctions compress and the dermal structure begins to collapse as results of skin cell turnover slows down leaving excess dead skin cell remaining on the surface. The visual results of intrinsic ageing are dry skin, fie lines, wrinkles, and sagging (Zaghi *et al*., 2009).

Extrinsic ageing known as photoageing, on the other hand, is caused by environmental factors such as cigarettes smoke, exhaust and pollution but the most common is over exposure to ultraviolet (UV) radiation (Krutmann, 2009) which induces photodamage (Alam and Havey, 2010). The caused damage of sun exposure not only blocks the ability of skin to repair itself but also continues to break down the synthesis of collagen. UV radiation also promote to degradation of elastin fibers causing the premature decrease in skin flexibility. The mainly visible sign of photoageing are hyperpigmentation, dry skin and deeply wrinkle (Giacomoni, 2005). A board spectrum of skin symptoms widely accepted as the result of reactive oxygen species (ROS) (Kaur *et al*., 2000; Saija *et al*., 2000).

To completely understand the molecular mechanisms responsible for photoageing in human skin, an awareness of the UV spectrum is important. Due to the UV spectrum is divided into three mainly levels; UVC (270-290 nm), UVB (290-320 nm) and UVA (320-400 nm) (Thiele and Dreher, 2005; Alam and Havey 2010). Ozone and atmospheric moisture filters UVC radiation, for this reason; never reaches through the earth. In conclusion, both UVA and UVB rays reach the physically surface. Photoageing is the superposition of UVA and UVB radiation on intrinsic ageing (McCullough and Kelly, 2005; Zaid *et al*., 2009). Because of biological effects on human skin, both types of UVA and UVB rays must be absorbed by chromophores in the skin. Deepening on the wavelength absorbed, UV light interacts with various

skin cells at different depth (Krutmann, 2009). More specifically, energy from UVB rays is mostly absorbed by the epidermis and affects epidermal cell such as the keratinocytes whereas UVA rays affect both epidermal keratinocytes and the deeper dermal fibroblasts. UVA absorption by chromophores mostly acts indirectly by transferring energy to generate ROS which causes several effects. On the contrary, UVB has a more direct effect on the absorbing chromophores. Approximately 50% of UV-induced photodamage is form the formation of free radicals (Alam and Havey, 2010).

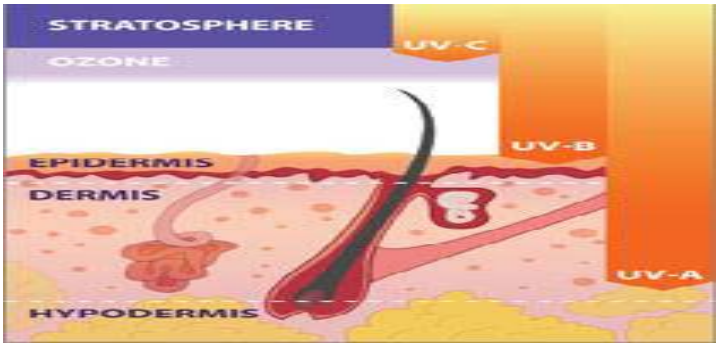


Figure 2 Schematic of UV penetrations through the skin

(From: http://amongthetortillas.com/2011/05/16/skin-cancer-part-2/)

As the previously data of ageing were reported, there are many theoretically hypotheses in order to explain based on scientific mechanisms of ageing in this decade. So the theory that can be obviously defined ageing in human is free radical theory remaining one of the most approve explanations of ageing at the molecular level (Schoneich, 1999). Free radical theory of ageing was firstly purposed by Harman in 1956, which basically indicated progressive ageing is associated with increasing steady state level of oxidatively modified biomolecules , hence; free radical reactions (Schoneich, 1999). Not only free radicals but non-radical reactive oxygen

also attack biomolecules, as a result; reactive oxygen and nitrogen species will be a preferred term to characterize the oxidants involved in such biomolecules modification. While a few free radicals such as melanin are not chemically reactive, most biologically-relevant free radicals are highly reactive. For most biological structures, free radical damage is closely associated with oxidative damage. Antioxidants are reducing agents, and limit oxidative damage to biological structures.

Biological ageing is a complicated process feathering species and tissue specific rates and molecular mechanisms of age related physiological and molecular changes (Masoro, 1993; Benzi and Moretti, 1995). Biological ageing is a function of several closely interrelated parameters; for examples, the metabolic rate, caloric intake, genetics, lifestyle and environment factors (Jazwinski, 1996; Sohal and Weindruch, 1996; Smith and Pereira-Smith, 1996; Finch and Tanzi, 1997; Morrison and Hof, 1997). Harman (1996) proposed the "Free radical theory of ageing" which generally implies that progressive ageing is associated with increasing steady state levels of oxidatively modified biomolecules as a result of free radical reactions. Free radical theory of ageing states that organisms age because cells accumulate free radical damage over time. A free radical is any atom or molecule that has a single unpaired electron in an outer shell (Buonocore and Groenedaal, 2007).

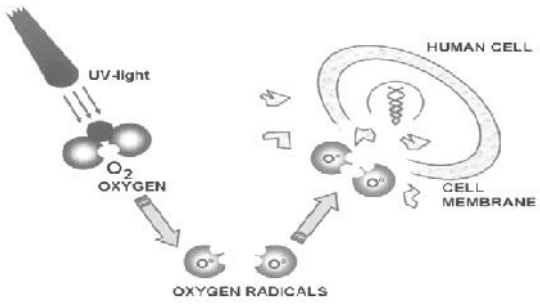


Figure 3 Representation of free radicals and cell damage (Morganti, 2009)

Reactive oxygen species (ROS) is one of the major types of free radicals. ROS is generated during normal aerobic metabolism (Buonocore and Groenendaal, 2007; Lau *et al*., 2009; Buonocore *et al*., 2010). Moreover, ROS production is normally neutralized by cellular antioxidant defense system so as to maintain equilibrium between the production and elimination of free radicals. UV radiation can induce ROS formation (Morganti *et al*., 2002; Blatt *et al*., 2005; Yaar and Gilchrest, 2007) being the cause generation in skin e.g. singlet oxygen and superoxide anion, promoting biological damage in exposed tissues via iron-catalyzed oxidative reactions (Yasui and Sakurai, 2003). These ROS enhance melanin biosynthesis, damage DNA, and may induce proliferation of melanocytes. A previous study (Yamakoshi *et al.,* 2003) also found evidence for a role of oxidative stress in pathogenesis of skin disorders. It is known that ROS scavengers or inhibitors such as antioxidants may reduce hyperpigmentation (Ma *et al*., 2001). To defense the skin against the deleterious effects as result of UV damage administration of natural antioxidants is basically required. Naturally antioxidant represents to function against the harmful effects from UV radiation.

Topical administration of enzymatic and non-enzymatic antioxidants is an excellent way of enriching the endogenous cutaneous protection system and therefore, an effective strategy for protection the skin against UV-mediated oxidative damage (Bonina *et al*., 1998). For a variety reason, natural antioxidants are providing for the potential as the active compounds as well. Traditional herbals are an interesting, largely unexplored source for development of potential new drugs. The ability of traditional herbals for development of new cosmetic skincare has been

emphasized recently (Kiken and Cohen, 2002). It is of great interest to know whether preparations used cosmetically through the wisdom have activities that might be useful in modern formulations.

2.4 Natural antioxidants

Antioxidants are fashionable (Le Bourg, 2005). The use of antioxidants in any anti-ageing skin care regimen is essential in order to combat and prevent further damage (Graf, 2005). The natural antioxidants are widely utilized for skin care as active and were documented as well during decades (Khaiat, 2000; Lau *et al*., 2009). Currently antioxidants are widely incorporated into a variety of cosmetic products. The commonly used antioxidants include vitamins A, B, C, E, CoQ_{10} and its analogues, minerals, and plants polyphenols such as tannins and flavonoids (Chiu and Kimball, 2003; Gerahty *et al*., 2010). New antioxidants are continually being explored while well known active compounds are improving continued researches. It is of interest to note that the possible of antioxidant plants as the active compounds combined with advanced technology.

Antioxidants from natural sources provide the efficiencies both for the treatment and prevention of oxidative stress (Radd, 2003; Thornfeldt, 2010). A board spectrum in term of activities of natural antioxidants was reported related in cosmetic. As common knowledge, natural antioxidants are favorable as skin care products; moreover, mainly sources were provided from synthetics and naturals (Zaid *et al*., 2009). The choice of synthetic or natural for cosmetic depends on their cost, safety, and effectiveness. More recently, a renewed interest in use of naturally occurring compounds is increasing consumption trend (Kapoor, 2009). Among the many

antioxidants that are now being formulated into topical products, natural antioxidants are interest.

2.4.1 *Phyllanthus emblica* Linn.

Figure 4 *Phyllanthus emblica* Linn.

(from: http://en.wikipedia.org/wiki/Phyllanthus_emblica)

Phyllanthus emblica Linn., synonym: *Emblica officinalis* Gaertn. also known as Indian gooseberry (Premi *et al*., 1998; Dnyaneshwar *et al*., 2006) or Ma-kham-pom in Thai (Mayachiew and Devehastin, 2008; Jaijoy *et al*., 2010) belongs to the Euphorbiaceae family (Majeed *et al*., 2009). It is an edible fruit (Kumaran and Karunakaran, 2006). This fruit also forms an important constituent of many Ayurvedic preparations as “Triphala”, for example. Emblica is widely distributed not only in tropical but also in parts of India, China, Malay Peninsula and Thailand region. Traditionally, the fruits of emblica are spaciously consumed raw, cooked or pickled (Kumaran and Karunakaran 2007) and have long been extensively used in folk medicine for treatment of several diseases; for instance, hemorrhage, jaundice, dyspepsia, etc (Scartezzini and Speroni, 2000; Kumaran and Karunakaran, 2006;

Raghu *et al*., 2007; Majeed *et al*., 2009). Recently, the potential of emblica fruits was well documented as the sources of vitamin C content (Premi *et al*., 1998; Scartezzini and Speroni, 2000; Kumar *et al*., 2006; Raghu *et al*., 2007; Raghu *et al*., 2007); for this reason, it is one of the top selling botanicals having diverse functional food in healthcare, food and cosmetic industry (Vohra and Gupta, 2005; Dnyaneshwar *et al*., 2006). Furthermore, extract of emblica was also reported to have antioxidant activity (Anila and Vijayalakshmi, 2000; Khan, 2009) in order to terminate free radical chain reactions in biological systems.

2.4.2 Chemical constituents

The wide variety of chemical constituents found in plants, many of them highly complex chemical structures, have been used as a biochemical resource by mankind (Cornuelle and Lephart, 2006; Liu *et al*., 2008). Polyphenols belong to heterogeneous class of compound possessing a variety of effects towards antioxidant activity (Hagerman *et al*., 1998; Thiele *et al*., 2000; Graf, 2005; Kumaran and Karunakaran, 2007). Emblica contains the different classes of constituents. The fruits of emblica enrich with hydrolysable tannins (Chaudhuri, 2005; Majeed *et al*., 2009) and other bioactive compounds. Tannins are considered as superior antioxidant; therefore, they are chemically complex substances widely distributed in the plant kingdom and employed in medicine as astringent (Chaudhuri *et al*., 2004). Pozharitskaya *et al*. (2007) reported that the antioxidant activity of emblica is the presence of ascorbic acid. In contrast to Ghosal *et al*. (1996) mentioned that emblica fruits not only contain ascorbic acid but also contain two hydrolysable tannins 10-12% (Chakraborty and Verma, 2010) having low molecular weight, namely, Emblicanin A and Emblicanin B (Zhang *et al*., 2001; Sairam *et al*., 2002; Yokozawa

et al., 2007; Khan, 2009). Baumann and Allemann (2009) purposed the emblica extract that acts at several different sites in the melanogeneis pathway. The two emblicanins had a very strong antioxidant action; moreover, they improved the efficacy of vitamin C (Scartezzini *et al*., 2006). Previously study demonstrated that aqueous extract of emblica contained high levels of polyphenolic compounds (Costa *et al*., 2010) such as gallic acid and ellagic acid (Damodaran and Nair, 1936; Summanen, 1999; Scartezzini and Speroni, 2000; Vohra and Gupta, 2005; Aqil *et al*., 2006; Yokozawa *et al*., 2007; Raghu *et al*., 2007; Ruangchakpet and Sajjaanantakul, 2007; Kalaiselvi *et al*., 2008; Lau *et al*., 2008; Shukla *et al*., 2009; Liu *et al*., 2008; Yokozawa et al., 2007; Pozharitskaya *et al*., 2007; Khan, 2009; Hazra *et al*., 2010). There is no report for the effect of emblica extract against UVB induced photo-ageing in human skin fibroblasts (Adil *et al*., 2010). In short, emblica has been shown to possess several pharmacological actions according to the publication.

Due to its antioxidant properties emblica extract has been shown to scavenge ROS, including superoxide anion, hydroxyl radicals and reactive nitrogen oxide radicals. Emblica extract has been reported to possess a wide spectrum of pharmacological properties including antioxidant, anti-inflammatory, anti-cancer and anti-microbial. Compounds such as phenolic acid can scavenge oxidative radicals, decrease lipid peroxidation and reduce the possible damage (Mestres *et al*., 2007). Among the active compounds naturally occurring in emblica extract, gallic acid and tannic acid are also reported to be the active constituents of phenolic compounds (Povichit *et al*., 2010; Jamuna *et al*., 2010) for use as the marker for study (Mahattanapokai, 2003; Kumaran and Karunakaran, 2006; Kumar *et al*., 2006;

Pozharitskaya et al., 2007; Luo *et al*., 2009; Majeed *et al*., 2009; Jaijoy *et al*., 2010). For this reason, gallic acid is the marker of bioactive compound in the study.

2.4.3 Bioactive compound from emblica extract

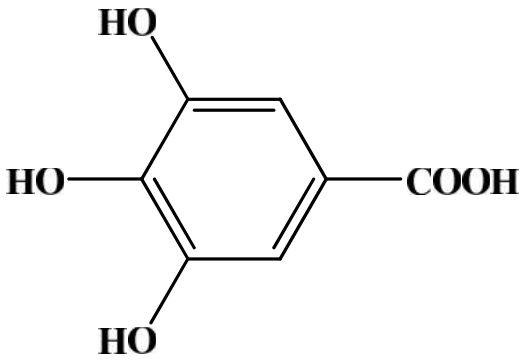


Figure 5 Structure of gallic acid

Gallic acid, naturally occurring is a trihydroxybenzoic acid, a type of phenolic acid (Pasanphan and Chirachanchai, 2008) and has also been reported to have antioxidant activity (Haslam, 1996). Moreover, polyphenols also act as depigmenting agents and inhibit UV-induced immunosuppression (Miteva and Fluhr, 2008). Gallic acid and its derivatives known as ellagic acid are biologically active compounds widely presented in plants and being starting material for hydrolysable tannins synthesis (Ossipov *et al*., 2003). It is strong antioxidant in emulsion or lipid system (Yen *et al*., 2002). In general gallic acid is widespread in plant food and beverages; for examples, tea and wine that was proven to be the anti-carcinogenic polyphenols presented in green tea leaf (Seth and Chand, 2000). Soong and Barlow (2006) reported that both gallic and ellagic acid are released by means of *in vivo* action of enzyme or acid that is easily hydrolyzed from hydrolysable from tannins. Pozharitskaya *et al*. (2007) isolated four compounds emblicanins A, emblicanins B,

gallic acid and ellagic acid from emblica extract by thin layer chromatography; moreover, they also implied that gallic acid was the production of ellagitannins by degradation process. According to gallic acid structure, it is polarity and hydrophilicity properties; for this reason, skin permeation and irritation will be occurred. An effective way to enhance cutaneous bioavailability of drug is to choose an appropriate delivery system (Mestres *et al*., 2007; Branka *et al*., 2009). Topical administration of natural antioxidants from plants could provide an efficient procedure to increase the endogenous cutaneous protection system. For this purpose, advanced technology in cosmetic as liposomes is the best candidate of choices in order to achieve both for *in vitro* and *in vivo* study (Nounou *et al*., 2006).

2.5 Liposomes, novel cosmetic delivery system

Liposomes are ideal system for cosmetic applications (Tadros, 2005); they were firstly discovered in 18^{th} century by Bangham (Lasic, 1998; Kulkarni, 2005). The advanced technology as liposome is the delivery system that provided significant benefits; for examples, enhanced delivery, improved active stability and increased efficacy of active ingredients (Memoli *et al*., 1994; Michniak and Wertz, 2005).

The term of liposomes encompass a board range of fatty acid organic compounds of plant and animal origin. A delivery system is the method of active transport onto the skin, pass through the lipid barrier and reaching the targeted lower layers beneath (Pollock, 2005). Delivery system would provide an alternative way to resolve the problems. For this reason, the key to achieve in delivery of the active and creation of the product is required for any actives. As a matter of fact, liposomes are

basically lamellar vesicular systems (Dayan, 2005). Sophisticated liposomes are microscopic spherical containers or vesicles (Hope *et al*., 1986; El Maghraby *et al*., 2008; Lasic, 2000; Cornell *et al*., 2010) with a diameter between 25-5000 nm (Draelos, 2010) formed as concentric biomolecular layers that are capable of encapsulation of drugs (Benson, 2005) mainly comprising of phospholipid (Pollock, 2005; Lui and Hu, 2007). As will become clear in the course of this review, liposomes can encapsulate many types of cosmetic agents (Strauss, 1989).

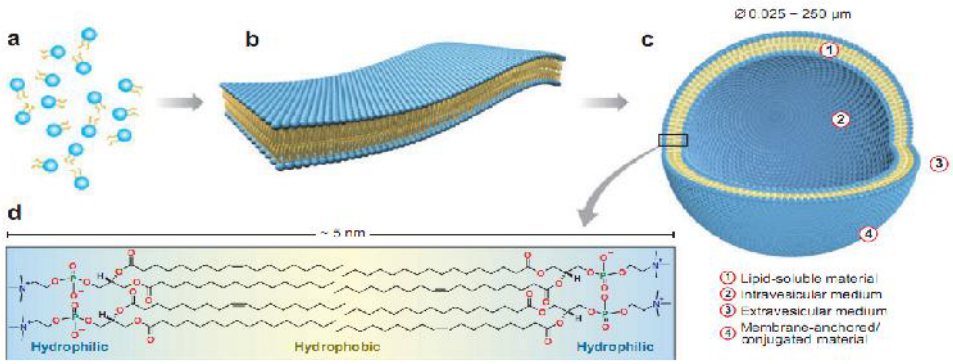


Figure 6 Structure of phospholipids and liposomes (Jesorka and Orwar, 2008)

Phospholipids are a class of lipid and a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline. The most common phospholipids are phosphatidylcholine (PC) molecules which are amphipatic molecules. Phosphatidylcholines are a class of phospholipid that incorporates choline as a head group (Benson, 2010a). They are a major component of biological membranes and easily obtained from a variety of readily available sources such as egg yolk or soy beans (Dayan *et al*., 2007). In general the structure of phospholipids comprises a hydrophilic head and a lipophilic tail as shown in Figure 6. In aqueous

solution they are arranged in bilayers. Liposomes are not only based on the natural structure of the cell membrane but also are primary formed from phospholipid, such as phosphatidylcholine originally from soy bean and egg yolk (Kaur *et al*., 2007). Their functionality may be influenced by chemical composition, vesicle size, shape, surface charge, lamellarity and homogeneity. Liposomes have a unique property of being able to encapsulate both soluble water materials within the aqueous compartment (Meier and Schreiber, 2005; Murthy and Shivakumar, 2010) and water insoluble materials embedded within the bilayer membrane (Nii and Ishii, 2005; Fang *et al*., 2007).

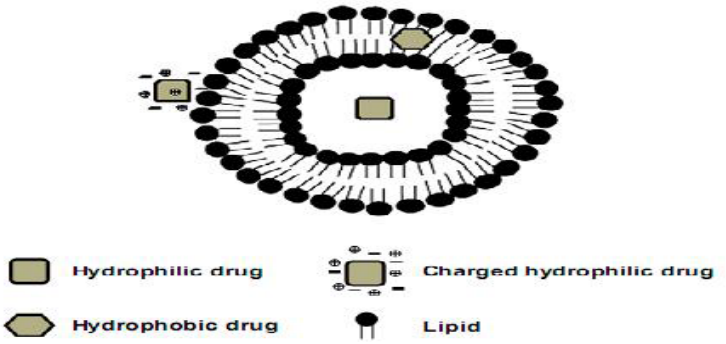


Figure 7 Representation of unilamellar vesicle displaying a single lipid bilayer with encapsulated molecules (Murthy and Shivakumar, 2010)

Liposomes can be characterized by size into several types approximately, such as small unilamellar vesicles (SUV), multi lamellar vesicles (MLV), and large unilamellar vesicles (LUV) (Kozubek *et al*., 2000) as shown in Figure 8.

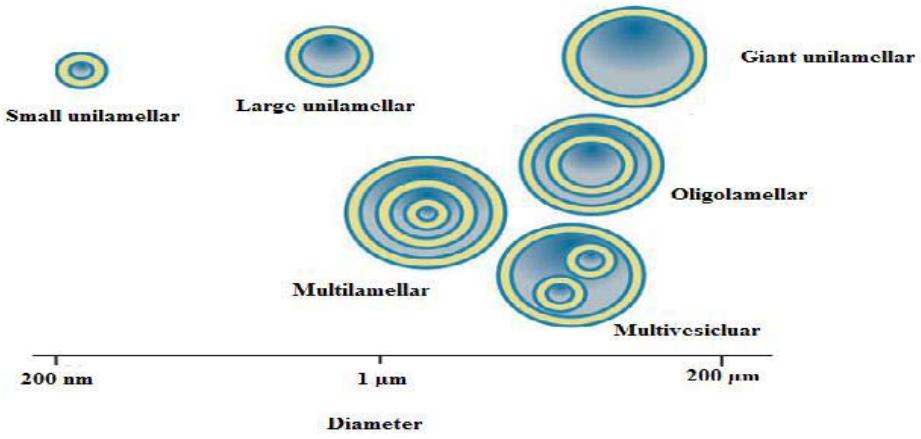


Figure 8 Classification of liposomes (Jesorka and Orwar, 2008)

2.5.1 Mode of action of liposomes

Certainly the concept of novel cosmetic delivery system is the methods of delivery active ingredients into the skin, by passing through the lipid barrier and finally reaching to the targeted site with minimize side effects (Michniak-Kohn *et al*., 2005). A board variety of the models for described interaction of delivery system with the skin is not clear. However, it is possible that liposomes either permeate the stratum corneum to some extent then interact with the skin lipids to release its active components (Naik *et al*., 2000; Benson, 2005b). Additionally, liposomes can occlude somewhat the skin surface and thus can increase tissue hydration (Gehring *et al*., 1997; Williams and Barry, 2006). In one of the very few published reports on the effects of mode of action , it is possible mechanism of transport and delivery would be fusing of the liposomes with the cell membrane (Kirjavainen *et al*., 1996; Fang *et al*., 2001), thereby allowing penetration into a skin cell (Zvulunov, 2009). In summary, liposomes remain confined to the upper skin layers, resulting in formation of active

reservoir mainly in the horny strata and also do not penetrate into the deeper layers of the skin (Verma *et al*., 2003ab). For this reason, liposomes could have efficient in local treatment of skin and for cosmetic uses (Touitou and Godin, 2006).

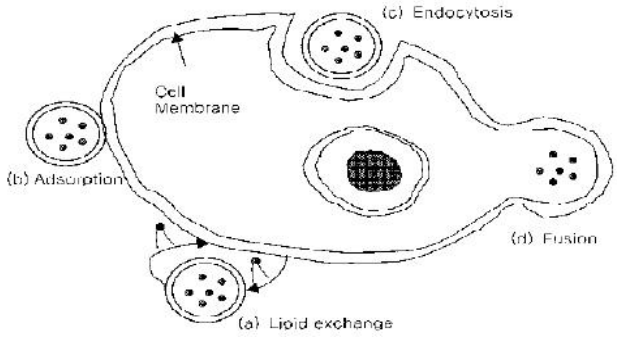


Figure 9 Interaction of liposomes with the cell membrane (Kim *et al*., 2003)

2.5.3 Application of liposomes in cosmetics

There are many beneficial of liposomal formulation; for instance, controlled released, reduce toxicity, increasing stability (Farrel and Sirkar, 1996; Stenekes *et al*., 2000; Elasayed *et al*., 2007; Cevc and Vierl, 2010). There is a considerable interest in the use of liposomes for cosmetic products that regard ageing or prevent photoageing. Antioxidants as natural botanical extract are also widely used in anti-ageing products (Arct *et al*., 2002), and for encapsulation of vitamins in liposomes have been shown to enhance the stability of vitamins. Compositions in liposomes are friendly to the skin.

Skin hydration

Liposomes as a carries itself offers advantages because lipids are well hydrated and also can reduce the dryness of skin which is the primary cause for ageing (Lasic, 1995). Liposomes have been shown enhance follicular delivery of both small polar molecules as well as macromolecules. It is possible that liposomes either

permeate the stratum corneum to some extent then interact with the skin lipid to release their active drug or that only their components enter the stratumcorneum. Several factors such as size, lipid composition, surface charge, mode of application have been reported to influence deposition into the deeper skin layers (Cornell *et al*., 2010). In order to maintain skin hydration, humectants are the key factor. The possible interaction is exchange of bilayer constituents, such as lipids, cholesterol and membrane bound molecules with components of cell membranes.

Controlled release

According to many reports dealing with standard liposomes concentrate on improved drug deposition through skin and its appendages, some early sources cited improved transdermal delivery (El Maghraby *et al*., 2008). Thus liposomes are an excellent formulation as drug and cosmetic carriers. Liposomes can be explored as slow release vehicles for drug delivery in extravascular regions such as skin by applying directly at the site of action (Gesztes and Mezei, 1988; Wen *et al*., 2006). According to the study of Patel (1984) mentioned that liposomes can be used for sustained release of drugs into the epidermis when applied topically.

Enhanced skin permeation

Enhanced skin penetration is one of the major interests for cosmetic treatments so liposomes have shown a good property. Based on results obtained with confocal laser scanning microscopy, van Kuijk-Meuwissen *et al*. (1998) concluded that liposomes are more favored than micellar solutions for achieving increased drug penetration through the skin. Their study also reported that when a lipophilic substance applied to skin via liposomes in a liquid crystalline penetrated deeper through skin than the same substance applied in the gel of liposome. Liposomal

tretionin gel (prepared with Carbopol 934) has produced a significant improvement in the treatment of acne (Sinico *et al*., 2005).

Low toxicity to skin

The components of liposomes are usually intrinsic components of organisms, so liposomes are of little toxicity and biodegradable. They are also not an irritant to skin. Glycolic acid is commonly used in many cosmetic products as an exfoliant and moisturizer. Unfortunately, the greater glycolic acid is the potential for skin irritation leading a burning sensation. Gupta (2003) reported that liposomes loading with glycolic acid improved for the controlled release and avoided applying glycolic acid through the skin.

Improving stability

In cosmetic, stability is one of the achievements. The study performed by Manosroi *et al* (2002) showed that tranexamic acid liposomes were stable double layers of the mutilamellar vesicles. Then, they also reported that liposomes containing tranexamic acid were relatively stable, as more than ninety percentage of total drug remained for two months.

2.5.4 Use of liposomes in cosmetics

Now nanotechnology was used in drug delivery system and cosmetic interest as advanced technology. For the commercial products, the first liposomal product was launched in 1986 by Christain Dior (Capture™) for anti-ageing cream (Kaur *et al*., 2007; Mu and Spando, 2010). It ranges from simple liposomes formulation as pastes which are used for replacement for conventional creams, gels and ointments for cosmetic products to formulations containing various extracts,

moisturizers (Lasic, 1995). Liposomes are used in cosmetic application or for transdermal delivery with the expectation that their use will result in an increase in the concentration of active ingredients. One of reasons for the widespread use of liposomes in cosmetic arena is their ease of preparation and the ability to improve the absorption of active ingredients via the skin. Dosage forms typically emulsions are popularity used as topical formulation (Soriano *et al*., 2001; Kanouni and Rosano, 2005; Palefsky, 2010) due to their widely potential applications. There are several types of emulsion that functions as a vehicle and delivery system for cosmetic.

2.5.5 Strategic formulation

A unique aspect of cosmetic formulations is the appearance of the final products: innovation in the formulation is not only by the performance of the active components, but also by the physiological impact of the appearance of cosmetic vehicle on the consumer (Magdassi, 1996). In skin care or cosmetic products, liposomes are formulated in suitable forms such as creams, gels, serums, lotions (Magdassi, 1997; Kulkarni, 2005; Kaur *et al*., 2007). It is well documented that the primary vector for topical formulation of active ingredients is a semisolid or emulsion. The main reason for selection of this dosage form is convenience. Emulsions are convenient because of typically two phases (hydrophilic and hydrophobic) (Cornell *et al*., 2010). As previously reported about beneficial liposomes, there is considerable interest to use of liposomes for products and retard premature ageing of skin, or prevent photoageing. Natural antioxidants are also used in cosmetic products; for examples, anti-ageing and skin lightening, and encapsulation

of vitamins. Liposomes have been proven to protect certain antioxidants, increase their bioavailability and provide release in sustained manner (Kulkarni, 2005).

The study of clinically efficacy of emblica extract alone or as a part of polyherbal formulations are used to combat signs both anti-ageing and skin whitening (Khan, 2009). More previously studies of emblica extract are published *in vivo* evaluation as follows;

Chaudhuri *et al*. (2004) recommended that the emblica extract could be used in formulations ranging from 0.1% to 1.0% (w/w) level. For antioxidant formulas used in 0.1 - 0.2% and 0.3 – 1.0% for age defying application. The pH of the formulation must be acidic (below 5.50) to maintain its antioxidant activity and stability. Penetration enhancer; for instance, lecithin may improve the extract's efficacy, in addition. It was previously discovered that significantly enhanced delivery of drugs through the skin could be obtained by using a novel permeation enhancing carrier.

Mahattanapokai (2003) evaluated emblica extract creams for 20 volunteers at various concentrations at 1 and 3 % of emblica extract that applied on one side of the face while cream base was applied on another side of the face. The results showed that 1% emblica extract cream could not be effective in improving skin moisture, skin lightening and skin roughness compared to the control whereas 3% emblica extract cream affected in improving skin lightening compare to the conventional cream base; moreover, it could not be effective in improving skin moisture and skin roughness as well.

Sujarit *et al*. (2007) studied the safety and efficacy of emblica cream and mulberry cream in 13 Thai female volunteers. In this study, in order to evaluate

both emblica cream and mulberry cream; briefly, the emblica cream was applied on the right bottom cheek site while mulberry cream was applied on the left bottom cheek specification amount and area for once daily, 0.1 g, 2.5 x 2.5 cm^2 respectively. The selected test application sites were photographed before the study and after 2, 4 and 6 weeks. The results of this study shown that the efficacy of emblica cream and mulberry cream in the melanin content was decrease in the second week (p=0.004 and <0.001) while the tendency of melanin content was increase in the fourth and sixth week. Furthermore, the elasticity and moisture of skin were not different of two products; whereas, sensory evaluation in the criteria of skin permeation, odor, viciousness, compatible of products and skin moisture and skin transparency after use were not different both emblica cream and mulberry cream as well.

Chaudhuri (2005) evaluated the potential of emblica extract as a safety data to produce phototoxicity by UVA irradiation on 20 human volunteers from a single dermal application. Under the conditions of the study, the test substance is classified as non phototoxic when tested on human volunteers at 2% dilution in distilled water. The result was reported that not a single volunteer showed any adverse effects.

Chaudhuri *et al*. (2004) studied the emblica as antioxidant to reduction UV-induced erythema that compared the emblica cream at 0.2 and 0.5%, 0.5% MAP (magnesium ascorbyl phosphate) and 0.5% vitamin E (natural tocopherol) on the back of human volunteers test sites area 4 x 2.5 cm, at a dose of 2 mg/cm^2. In short, the volunteers were divided 2 groups; for group A 11 volunteers were applied product for 8 days and then on day 9 induced pigmentation by UV light that compared the untreated irradiated control site versus the product-treated sites on day 10. For group

B 10 volunteers were induced pigmentation by UV light then immediately applied the product and continued product application once a day for 10 days which compared the untreated irradiated control site versus the product-treated sites. The result showed that, a statistically significant (p<0.05) only reduced in erythema with 0.2% emblica cream, 0.5% vitamin E cream in protocol A. In protocol B, only 0.2% emblica cream showed statistically significant reduction in erythema.

Chaudhuri *et al*. (2007) performed 6 clinical studies healthy male and female demonstrating the effect of emblica on normal skin on upper left and right arms for 9 weeks. Each volunteer given 2 products comprising emblica extract lotion on upper left arm the other one as positive control on upper right arm; next, was instructed to apply approximately 0.5 mL of the test materials twice daily. The tested sited areas were photographed before the study then after 3, 6 ending in 9 weeks. All experiments were separated into 6 studies as summarized in the table 1.

Table 1 Clinical studies of emblica on normal skin

Study of the experiments	Results
Study 1: 2% emblica lotion vs. 2% hydroquinone lotion (13 Asians) Study 2: 2% emblica lotion vs. 2% hydroquinone lotion (13 Hispanics) Study 3: 1% emblica lotion vs. 3% magnesium ascorbyl phosphate lotion (16	1. The result of the study 1 and 2 clearly showed that 2% emblica lotion has an effective to be skin lightening versus 2% hydroquinone in Asian as well as Hispanic volunteers. (two-tailed p<0.001) 2. Study 3 showed that 1% emblica lotion was at least three times better in skin lightening efficacy on a percentage active

Asians) Study 4: 1% emblica lotion vs. 2% kojic acid (16 African-Americans) Study 5: 1% emblica lotion vs. 3% ascorbyl glucoside lotion (14 Hispanics) Study 6: 1% emblica lotion vs. 0.1% licorice root extract lotion (15 Hispanics)	basis over the 3% magnesium ascorbyl phosphate lotion. (p<0.01) 3. Study 4 and 5 showed that 2% kojic acid and 3% ascorbyl glucoside was better than 1% emblica lotion and, respectively. (p<0.001) 4. Study 6 showed that 1% emblica lotion was not different to 0.1% licorice root extract.

Moreover, Chaudhuri *et al*. (2007) also performed clinical study *in vivo* skin lightening on 19 Caucasian healthy female volunteers. This study was carried out for 8 weeks, the application site on the entire face twice daily; indeed, each volunteer was given lotion containing 1% emblica extract. The Chroma Meter CR-321, from Konica Minolta the device was used as skin color measurement; after that, the selected test application was evaluated after 4 and 8 weeks. The result showed that a statistically significant (p<0.005) reduction in freckle spot was seen especially after 8 weeks in 17 out of 19 volunteers.

2.6 Instrumental principle and measurement for evaluation of skin

Nowadays, various methods have been introduced to assess the skin conditions for quantitative evaluation of several parameters related into skin function (Berardesca, 1997; Piérard, 2002). Without any instrumentations, it is impossible to determine what the product is really doing, altering on the skin (Khazaka, 2011). Evaluation of the skin structure and functional properties of cosmetic claims on the

efficacy skin care product is a key role for research and development (Chiu and Kimball, 2003; Wickett *et al*., 2007). The use of digital photographs of volunteers combined with image analysis has provided clinical investigators in wrinkles, hyperpigmentation, pore size, skin tone and other skin conditions (Stephens *et al*., 2010). Most scientists would agree that the use of non-invasive methods is one way for generating quantitative data about a product's performance on skin (Khazaka, 2011). Due to the fact that is not only for the evaluation of the skin but also for the measurement parameters are essential to cosmetic section, for examples; hydration, sebum, pH of skin, elasticity and biological skin ageing, skin thickness, skin structure. The measurement of skin can be performed by several devices in order to measure skin parameters to supplement clinical grading. Though the use of technical device to perform efficacy testing and claim support in the cosmetic segment has become an indispensable and established standard. Later, consequently, the first commercial available skin measurement devices were provided to the market. This trend has contributed to the ability of manufacturers of cosmetic to improve their product quality and also the confidence of the consumer in choosing for the right product of their specific needs as well (Khazaka, 2011). At present, there is a variety of cosmetic device available for different parameters that should be self-governing for institute. The measurement principles differ considerably depending on the parameter and manufacture of the instrument.

In this study, Etude Skin Counseling System, Moritex, Japan is the non-invasive device to measure objectively a variety of skin parameters. Etude skin counseling system is a complete system for cosmetic skin counseling offering measurement, analysis and result presentation to assist counseling and

recommendation of skin care products. The results can be stored and recalled for comparison at later sessions.

(a) (b)

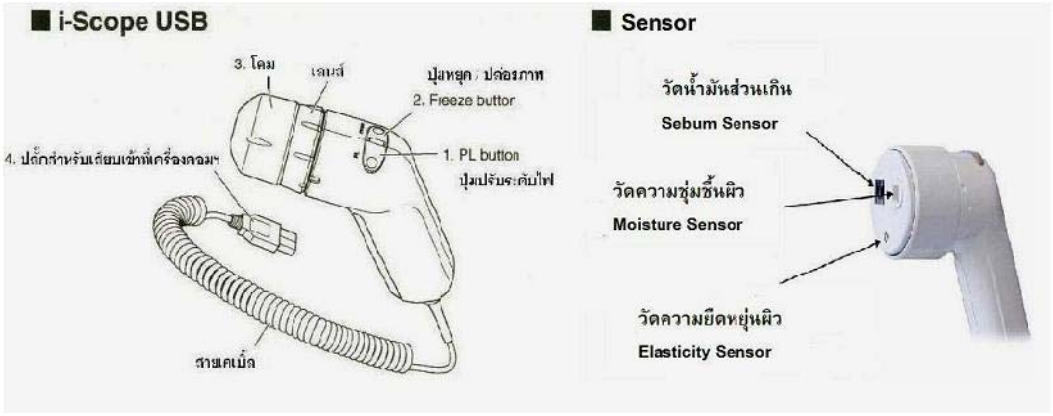


Figure 10 Etude Skin Counseling System (a): i-scope (b): sensor

The system composes of i-Scope and Sensor as shown in figure 10. Presentation material and data can be customized to your individual requirements. It features two mainly parts comprising i-Scope and Sensor that are different functions. The feathering of i-Scope evaluates the skin texture, dullness and squames. Topical products that are generally measured in cosmetic are the following:

2.6.1 Skin texture

The skin texture is the appearance of the skin smooth surface. To the features of this texture, many factors are occurring, for instance diet and hydration, amount of collagen and hormones. A gradual decline in skin is moreover superimposed by age. As skin ages, it becomes thinner and more easily damaged, with the appearance of wrinkles. The deterioration is also accompanied by a darkening of skin color for an over absorption of the natural coloring pigment, melanin on the top

most cell layer in skin. The skin texture also depends on its body location. Skin texture was evaluated into two parameters such as fineness and orientation. In the case of image processing, texture appearance should be considered in changing with image recording parameters, that is illumination and direction of view, a problem common to any real surface.

2.6.2 Skin dullness

Skin color is primary determined by melanin, produced by melanocytes of the basal layer of the dermis which comprises two mainly parameters; transparency and degree of pigmentation. Melanin also defines as the degree of pigmentation. In lighter skin types, the introduction of UV light influences color. A secondary contributor to skin color is the hemoglobin located in the blood capillaries of the dermis. It appears as a representative of blood perfusion and depends on the amount of oxygenated (red) and deoxygenated (blue) blood which can be influenced by temperature and emotions. Moreover, determining skin color is an important factor in selection of make-ups as well as in skin whitening skin care. Reflected red, green, blue and yellow light measured by photometric sensor is correlated to a given set of existing colors.

The genetically determined intrinsic degree of pigmentation can be influenced by external factors like free radicals and UV light, causing sun damage and spot, which can accelerate the normal ageing process. This can influence the degree and distribution of melanin in the skin as a result of uneven pigmentation; such as, age spots. Visible, later stage pigmentation can be seen under polarized light and early stage, barely visible spots can be seen by means of UV light.

The device is based on the difference of absorption of melanin and hemoglobin has a peak light absorption at 560 nm (green light) and also absorbs slightly in wavelength range of 650 to 700 nm (red light). The photo detector measures the light reflected by the skin at wavelength in the green and the red for hemoglobin and for melanin, respectively. The melanin index (M-index) is mainly influenced by the melanin content, whereas the erythema index (E-index) is influenced by the hemoglobin content; in short, assuming that absorbance of hemoglobin and melanin is similar to the wavelengths for green and red light. Results are then converted and displayed as colorimetric values in the three dimensional L*a*b* system, as determined by the Commission Internationale de l Eclairage (CIE) (Galzote *et al*., 2008; Holm, 2011). The nature of human color vision has been quantified for the propose of color measurement in terms of three color matching functions *x*,*y*,*z*.

2.6.3 Keratinization

It has been known for a long time that desquamation mechanism is disturbed by several factors, for instance, psoriasis, ichthyosis and some cosmetic problems related to dry skin (Lévêque, 1999). Squamometry consists of sampling the most superficial layers of the stratum corneum by means of a sticky tape firmly pressed onto the skin (Paye and Cartiaux, 1999; Paepe *et al*., 2001). In the present study, the correlation between stratum corneum hydration and scaling of the skin was investigated using capacitance measurements and squamometry, respectively.

(a) (b) (c)

Figure 11 (a): skin texture, (b): skin dullness and (c): keratinization

The values obtained are reported as percentages, and ranked as low, medium and high, in comparison with reference values in a population of the same age range provided by the manufacturer. Texture, dullness and keratin were scored as A, B, or C (good, fair or poor, respectively) when compared with references values.

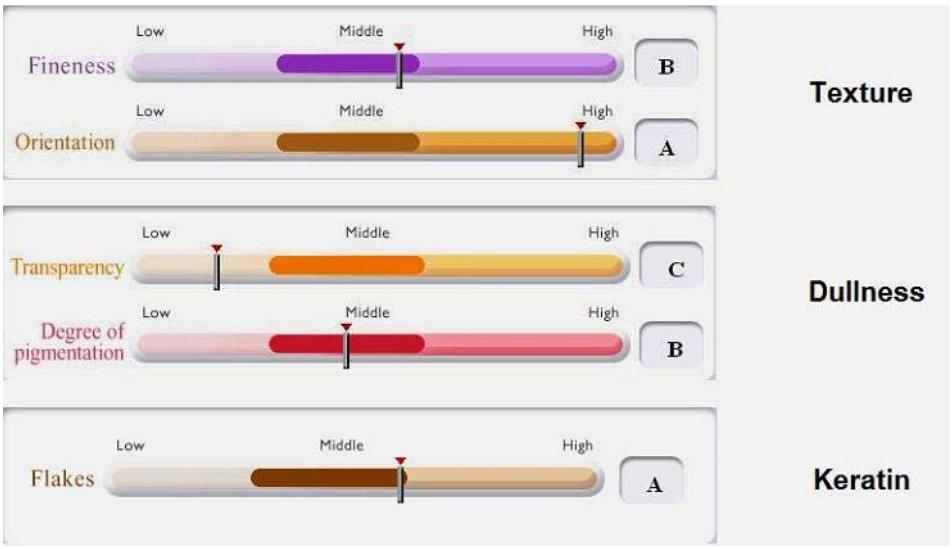


Figure 12 Evaluation of skin parameters; Texture, dullness and keratin

2.6.4 Skin hydration

While assessment of skin by clinical grading is still important to both moisturizer and mildness studies, there are now many noninvasive methods to supplement and enhance the evaluation of skin. To achieve in skin hydration

assessment, available methods range from evaluation of barrier integrity to measurement of water loss, infrared spectroscopy, magnetic resonance imaging, skin surface topography, scaling of the skin surface (Wickett *et al*., 2007). The choice of method depends upon the characteristics of the skin to be quantified.

As some water from the epidermis must be the present to provide sufficient hydration for both stratum corneum flexibility and the functioning of enzymes that are important to stratum corneum barrier function and desquamation (Wickett *et al*., 2007). Moreover, the water content of the stratum corneum influences other skin features, such as barrier formation, drug penetration, skin softness and skin elasticity (Galzote *et al*., 2008).

2.6.5 Sebum

Face is covered by lipid film derived from sebum and epidermal lipid which is secreted by the sebaceous glands. Facial sebum secretion is an important consideration in cosmetic skin care. Both excessive sebum secretion and reduced sebum secretion are cosmetically undesirable (Youn *et al*., 2005).

Sebum production plays an important role in skin hydration by producing glycerol, which is necessary for an intact skin barrier. Moreover, sebum supplies lipids to the surface of the epidermis that may aid in preventing transepidermal water loss (TEWL) (Elsaie and Baumann, 2009). Excess sebum production produces oily skin, and in many cases contributes to acne.

2.6.6 Elasticity

There are many reasons to investigate the mechanical properties of the skin *in vivo*. In cosmetic, skin elasticity is determined by the elastin and collagen fibers of the dermis. Due to ageing and external factors, the elasticity of the skin

deteriorates over time, leaving the skin appearing dull and saggy. The elasticity sensor applies measurement technique where a tiny sensor tip oscillates at a particular frequency and, when applied to the skin, will exhibit a change in the frequency reflecting the firmness of the skin.

For sensor, skin parameters; moisture, sebum and elasticity were ranked in three levels low, medium and high as shown in figure 13. Relative moisture values correspond to a scoring scale from 0 to 99. In addition to moisture, this sensor evaluates the amount of sebum on the particular area of the skin which can be evaluated resulting skin type (oily, oily-dry, dry, or normal).

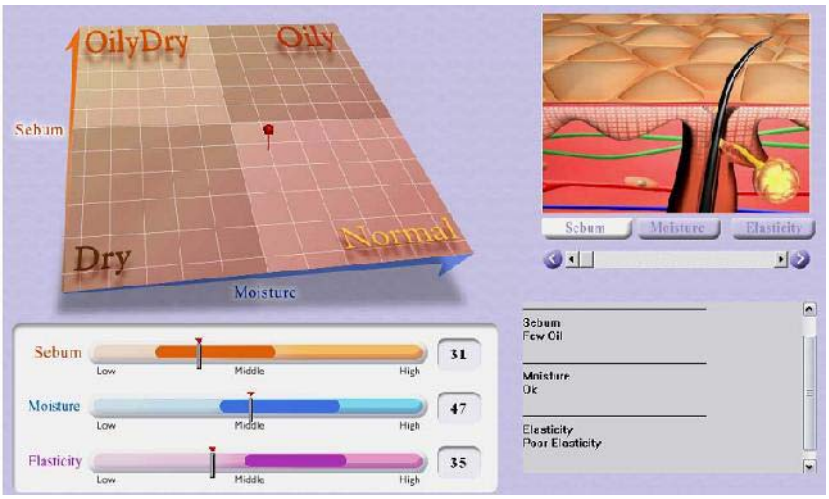


Figure 13 Evaluation of skin parameters; sebum, moisture and elasticity

Etude skin counseling system is important devices in esthetic skin since it gives fast, relevant results for the key factors determining the actual skin conditions. Analysis with scoring before and after treatment can even document progress over time, which is the key achievement for satisfaction.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Emblica fruits used in this study were collected from Krabi Province, Thailand in February 2008.

3.1.2 Chemicals and reagents

Absolute ethanol, AR grade (Merk®, Darmstadt, Germany)

Acetic acid (AnalaR®, BDH Labatory, England)

Acetonitrile (Lab-Scan®, Thailand)

Ascorbic acid (Riedel-deHean®, Seelze, Germany)

Deoxycholic acid (Fluka®, Buchs, Italy)

2,2-diphenyl-l-picryl-hydrazyl (Sigma-Aldrich®, Germany)

di-Sodium hydrogen orthophosphate (Univar®, Ajax Finechem, Australia)

Ethanol 95% (Merk®, Darmstadt, Germany)

Folin & Ciocalteau's phenol reagent (Fluka®, Buchs, Switzerland)

Gallic acid, AR grade (Sigma®, St.Louis, USA)

Methanol (Lab-Scan®, Thailand)

3-sn-Phosphatidylcholine from soy bean (Fluka®, Buchs, United States)

Sodium acetate (Univar®, Ajax Finechem, Australia)

Sodium chloride (Carlo Erba, Mantedsion Group)

3.2 Instruments

Table 2 Experimental instruments

Instrument	Model	Company
Centrifuge	Z323K	Hermle Labortechnik GmbH, Germany
Chromatographic column	BDS HYPERSIL C_{18} column 250 x 4.6 mm, particle size 5 µm	Thermo United States
High performance liquid chromatography	SpectraSystem P1000 pump SpectraSystem UV 1000 detector ChromQuest software program SN4000	Thermo Electron, United States
Homogenizer	PT 1200E	Polytron, Switzerland
Modified Franz diffusion apparatus	57-6M	Hanson Research Corporation, United States
Rotary evaporator	Eyela N-1000 series	Tokyo Rikakikai Co.,Ltd., Japan
Sonicator	HT Crest	S.V. Medico Co., Ltd., Thailand

Ultracentrifuge	Optima L-100XP, equipped with SW 60 Ti rotor	Beckman coulter, United States
Vortex mixer	G-560E	Scientific Industries Inc., United States

3.3 Methods

3.3.1 Preparation of crude extract

The freshly harvested fruits of emblica were unwrapped, dried at 50 °C and grinded to powder. The grinded ground fruit materials were soaked in 95% ethanol for 3 days. The extract was filtered through nylon cloth and the re-extracted was performed twice with the same solvent. The maceration was repeated 3 times to get total crude extract. All filtrates were collected and concentrated in a rotary evaporator. The percentage of yield was calculated.

3.3.2 Preformulation studies

3.3.2.1 Determination of antioxidant activity

Radical scavenging activity of the extract against stable DPPH (2,2-diphenyl-1-picryl-hydrazyl) was determined using method of Williams *et al*. (1994). In short, the stock solutions of the extract were prepared in ethanol (1 mg/mL). The extract was further diluted with ethanol for at least 5 dilutions (0.2, 0.4, 1, 2 and 4 µg/mL). The solution of DPPH in absolute ethanol ($6x10^{-5}$ M) was prepared daily.

100 µL of DPPH solution were mixed to an equal volume of various concentrations of the extract solutions in 96-well plate reader at final concentrations of 0.1, 0.2, 0.5, 1 and 2 µg/mL. The mixture was shaken vigorously and kept in the dark at room temperature for 30 minutes. The absorbance of mixture solution was measured at 517 nm by spectrophotometer. Absolute ethanol (100 µL) plus emblica extract solution (100 µL) were used as a blank, while DPPH solution plus absolute ethanol was used as a control. All determinations were performed in triplicate and the results were averaged. In this experiment, a synthetic antioxidant regent, ascorbic acid, was used as a positive control in the same system. Radical scavenging activity was expressed as the percentage of DPPH inhibition calculated by the following equation:

$$\% \text{ Inhibition} = \left(\frac{Ab_{control} - Ab_{sample}}{Ab_{control}} \right) \times 100 \qquad (3.1)$$

$Ab_{control}$ is the absorbance without test solution at 517 nm.

Ab_{sample} is the absorbance with test solution at 517 nm.

The 50 percent inhibitory concentration (EC_{50}) is the concentration of substrate that scavenges the DPPH radical by 50 percent. This was calculated from the plot of graph between concentrations of emblica extract and percent of DPPH radical scavenging activity.

3.3.2.2 Total phenolic content

The total phenolic content in the emblica extract was determined by the Folin-Ciocalteu method described by Miliauskas *et al*. (2004) with minor modification. The Folin-Ciocalteu method is an electron transfer based assay. It measures reducing capacity which is corresponding to the phenolic content of biological materials. This method is based on the fact that phenols ionize completely under alkaline conditions and can be readily oxidized by the Folin-Ciocalteu reagent. This oxidative reaction gives the blue complexes of phosphomolybdic/ phosphotungstic acid which can be determined spectroscopically at 765 nm.

A solution (100 µg/mL) of the extract was prepared in 95% ethanol. 20 µL of this solution was then mixed with 100 µL of 0.2 M Folin-Ciocalteu reagent (10-fold dilution) and 80 µL sodium carbonate (75 g/L). Its absorbance at 765 nm also was measured after 30 minutes incubation at room temperature. Absolute ethanol plus Folin-Ciocalteu reagent and sodium carbonate was used as negative control. Each sample was performed in triplicate. The concentration of the total phenolic compounds in the test sample was calculated from calibration curve of gallic acid.

Calibration curves were constructed using 5 standard solutions of gallic acid which covered a concentration range of 5, 8, 10, 20, 30, 40 and 50 µg/mL. Absolute ethanol plus Folin-Ciocalteu reagent and sodium carbonate was used as a blank. All determination was performed in triplicate. The calibration curve was constructed by plotting absorbance versus gallic acid concentration.

The content of total phenolic in the emblica extract were expressed as gallic acid equivalents (GAE) which refers the phenolic content as the amount of

gallic acid in milligrams per gram of the extract. These values were calculated by the following formula:

$$C = c \times (V/W) \quad (3.2)$$

Where; C: total content of phenolic compounds (mg GAE/ g of the extract)

c: the concentration of gallic acid established from calibration curve (µg/mL)

V: the volume of extract (mL)

W: the weight of plant extracts (mg)

3.3.3 High Performance Liquid Chromatography (HPLC) analysis

3.3.3.1 Validation of HPLC method

The HPLC method used for analysis of gallic acid was validated in terms of linearity, accuracy and precision according to International Conference on Harmonization (ICH) guidelines (ICH, 1996) which should be considered as follow:

Linearity

A linear regression equation applied to the results should not have an intercept significantly different from zero. The peak area of gallic acid was plotted against the concentration of gallic acid. A linearity least-squares regression analysis was conducted to determined slope, intercept and coefficient of determination (r^2) to demonstrate linearity of the method.

Accuracy

Accuracy is the measure of exactness of an analytical method and is measured as the percentage of recovery. The samples were spiked with three different amounts of standard compounds.

Precision

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements both the linearity and the recovery data for the statistic assessment. From the perspective of the designated, these validation data should be noted as intra-day precision and intermediate precision, inter-day precision.

Repeatability

The ability of the test method to generate the same results over a short time interval under identical condition should be determined from a minimum of nine. The intra-day accuracy and precision were assessed from the results of three replicate analyses of quality control sample with three different concentrations: low, medium and high on a single assay day. The inter-day accuracy and precision were determined from the same quality control samples analyzed on three consecutive days. Precision of the assay was expressed as percent relative standard deviation while accuracy was expressed as the percent bias which is the relative different between the measured and true value.

3.3.4 Quantitative of emblica extract by HPLC

The standard calibration curve was determined from standard solutions of gallic acid which concentration range 1, 2, 4, 10, 20, 30, 40 and 50 µg/mL. All determinations were performed in triplicate. Aliquot of standard solutions were injected into HPLC system. The standard curve was then constructed by plotting the peak area versus gallic acid concentrations. The analyte peak values were plotted against the corresponding concentrations expressed as µg/mL.

The amount of gallic acid in the emblica extract was measured using HPLC method described by Kumaran and Karunakaran (2006). The solution was filtered through 0.45 µm nylon syringe filter. The filtrate was injected into HPLC with the following conditions;

Column	: Reverse Phase BDS HYPERSIL C_{18} column (250 x 4.6 mm, particle size, 5 µm)
Mobile phase	: 0.05% H_3PO_4 (concentrated, 85%) in water (A) and acetonitrile (B)
Flow rate	: 1.0 mL/min
Injection volume	: 20 µL
Detection	: UV 220 nm
Gradient program	: 0-6 min 5% B, 6-15 min 15% B, 15-35 min 20% B, 35-40 min 40%

3.3.5 Preparation of the emblica liposomes

Liposomes containing emblica extract obtained from Nobnorb (2008). The amount of emblica extract in liposomal formulation was 1% w/v, which was optimized. The ingredients used for preparation are listed as following:

Lipid:

Phosphatidylcholine from soybean (SPC)

Additive:

Tween 80

Surface charge:

Anionic, Deoxycholic acid (DA)

Preservative:

Uniphen P-23

Method of preparation

Liposomes were prepared by modified ethanol injection method. Each material was accurately weighed and placed into two separate vessels, one for the water phase and another for the lipid phase. The water phase consisted of 1% w/v emblica extract in a mixture of 95% ethanol/acetate buffer pH 5.5 solvent mixtures (50:50 v/v). The lipid phase consisted of mixture of lipid, additive, surface charge and preservative in 95% ethanol. The temperature of both phases was control at 60 $^{\circ}$C before mixing. Liposomes formed spontaneously after the ethanol was removed with a rotary evaporator leaving behind the suspension. Subsequently, the emblica liposomes were transferred into a glass bottle, sealed and characterization.

3.3.6 Characteristics evaluation of emblica liposomes

Particle size

Photon correlation spectroscopy (PCS) was used to measure the diameter of emblica liposome. The emblica liposome was analyzed at 25ºC after dilution of dispersion to an appropriate volume with distilled water. The measurements were carried out in triplicate.

Encapsulation efficiency

To determine the amount of emblica extract encapsulated in liposome vesicles, the unencapsulated extract was first separated from the liposome dispersion using ultracentrifugation technique.

Testing procedure and data analysis

The sample of 4.0 mL liposomes suspension was loaded into a centrifuge tube. The liposomes suspension was centrifuged at 60,000 g and 4°C for 2 hours controlled conditions, in order to separate the incorporated emblica from free emblica liposomes. The content of gallic acid of the unencapsulated extract was analyzed by HPLC method as describe in section 3.3.4 all measurements were performed in triplicate. Encapsulation efficiency (EE %) could be calculated from the following equation:

$$EE\% = \left(\frac{Total - Free}{Total}\right) \times 100 \qquad (3.3)$$

Where; Free : The total contents of the unencapsulated extract

Total : The total content of gallic acid in extract

3.3.7 Development of cream containing emblica liposomes

Formulation of cream bases

Oil in water emulsion cream bases were formulated with different materials and emulsifying agents as shown in Table 3. All cream bases were prepared by beaker method. Each material was accurately weighed and placed into two separate beakers, one for the oil phase and another for the water phase. Then, they were heated to about 70-75°C in a water bath. Once the desired temperature was reached, the constants from the water phase were added into the oil phase with constant stirring. The mixture was stirred until it congealed at room temperature. All cream bases were evaluated under a heating and cooling cycle tests (45°C, 24 h and 4°C, 24 h; 6 cycles). Their physical appearances including color, smoothness, phase separation and pH were observed before and after the stability testing. Cream bases with good appearances and promising stability were then selected for developing emblica creams.

Table 3 Lists of ingredients

Ingredient	Content (g)				Mode of action
	Rx1	Rx2	Rx3	Rx4	
Carbopol 934	-	-	-	0.75	Gelling Agent
Steareth-10	-	-	-	1.5	Emulsifying Agent
Cetyl Alcohol	-	-	-	0.5	Stiffening Agent
Unirepair T-43	1	1	1	1	Environmental factors
Arlacel 165	1	1	-	-	Emulsifying Agent
Cetostealyl Alcohol	1.5	-	1	-	Stiffening Agent
Cetoceryl Alcohol	-	1.5	-	-	Stiffening Agent
Stearic Acid	-	-	3	-	Stiffening Agent
Cremophor A6	-	-	1.5	-	Emulsifying Agent
Cremophor A25	-	-	1.5	-	Emulsifying Agent
Beeswax	1.5	1.5	-	-	Stiffening Agent
GMS SE	2.5	2.5	2	-	Emulsifier
Lanolin	1	-	-	-	Emollients
Petrolutum (Vassaline)	-	1	1	-	Emollients
Mineral Oil	3	3	2	1	Solvent
Isopropyl Palmitate	2	-	-	2	Emollients
Isopropyl Myristate	-	2	1.5	-	Emollients
Dimethicone	1	1	-	-	Antifoaming Agent
Sepigel 305	1.5	1.5	1	-	Emulsifying Agent
Tween 60	-	-	-	1.6	Wetting and/or Solubilizing Agent
Span 60	-	-	-	1	Wetting and/or Solubilizing Agent
Propylene Glycol	5	-	5	1	Solvent
Glycerin	-	5	5	4	Solvent
Squalane	2	2	-	2	Emollients
Disodium EDTA	0.1	0.1	0.1	0.1	Chelating Agent
Triethanolamine	-	-	-	0.75	Neutralizer
Paraben Concentrate	1	1	1	1	Preservatives
Purified Water to	100	100	100	100	Solvent

3.3.8 Stability of containing emblica liposome

Creams of emblica liposome were prepared using suitable cream bases from section 3.3.7 the emblica liposome was incorporated in cream bases after them congeal at room temperature. Concentrations of the emblica liposome in cream bases were 1% w/w. In addition, the creams containing 1% w/w emblica extract and 1% w/w of percent active blank liposome were formulated as control in this study.

Stability evaluation

Each of the cream formulation was evaluated for its physical stability for 3 conditions; under heating and cooling cycle tests (1 cycle: 4°C, 24 h and 45°C, 24 h) for 6 cycles. Moreover, the cream formulations were stored at room temperature, 45°C and 4°C for 3 months for monitoring the content of gallic acid that was the active compound in emblica extract. The physical appearances of cream formulations including color, smoothness, and phase separation were observed before and after the stability testing. In addition, the antioxidant activity of the cream formulations was determined and measured pH and viscosity before and after stability evaluation. The experiments were performed in triplicate.

Testing procedure for antioxidant activity of cream formulations

1 gram of each formulation was weight and mixed with 10 mL of methanol. After 30 minutes sonicating, it was centrifuged at 5,000 rpm at 4°C for 20 minutes. Then, the solution was filtered through 0.45 µm nylon syringe filter. The filtrate was determined for its antioxidant activity using DPPH radical scavenging assay as described in section

Testing procedure for gallic acid content of cream formulations

The gallic acid content in each formulation was determined using high performance liquid chromatography (HPLC) method the sample was extracted as the same method of antioxidant determination. All determinations were performed in triplicate.

3.3.9 *In vitro* skin permeation study

This study was performed in order to examine the potential of the emblica liposome cream as a transport delivery system of the emblica extract which is the hydrophilic substances into the skin. The *in vitro* skin permeation study was performed using modified Franz's diffusion cells and pig's skin as they represent skin model as followed SCCNFP (1999). The experiment was performed comparing to the controls including the cream base, 1% emblica liposome cream, blank liposome cream, 1% emblica extract cream, emblica liposome and blank liposome as well.

Testing procedure

The skin permeation studies were performed in Modified Franz's diffusion cells (Hanson Research) at 37ºC during 12 hours. Full-thickness new born pig skin was clamped between the donor and the receptor phases with a diffusion area of 1.77 cm^2. Pig's skins were cleaned and hairs were removed from the skin with clipper. Care was taken not to damage the skin surface. The skin both sides of newborn pig were cut open and lipid on skin was separated. The skin was stored in freeze at -20ºC of refrigerator until the permeation study. Before experiments were performed, the skin was incubated in phosphate buffer pH 7.4 (USP) for 2 hours. The receptor compartment was filled with 11 mL of phosphate buffer solution while the donor chamber was filled with 1.0 g of each formulation and covered with parafilm to prevent drug evaporation. The receptor solution was continuously stirred at 210 rpm, kept temperature at 37ºC by circulating water through an external water jacket and protected from light with aluminum foil. All determinations were performed in triplicate 1.0 mL an aliquot of solution in the receptor chambers was withdrawn through the sampling port at 0.5, 1, 2, 3, 6, 9 and 12 hours and the cells were refilled

with fresh phosphate buffer solution in order to keep the volume of receptor solution constant during the experiment. The antioxidant capacity of each sample was then determined by the DPPH radical scavenging assay and the solutions in receptor compartment were analyzed by HPLC method. Both of the antioxidant activity and HPLC method were performed in triplicate.

3.3.9.1 Analysis of gallic acid retained in skin

The quantification of drugs within the skin is essential for topical and transdermal delivery research. So, a determination of drug concentration within whole skin requires removal of the excess formulation, follow by extraction in a suitable solvent (Touitou *et al*., 1998; de Hassonville *et al*., 2004). At the end of the *in vitro* transdermal diffusion experiments of the amount of gallic acid retained in the skin was extracted by cutting the pig skin with the scissors to small pieces and soaked in methanol 5 mL then homogenized at 24,000 rpm for 5 minutes. Filtered the small pieces of pig skin with the filtered paper and centrifuged at 15,000 rpm for 30 minutes at 4ºC. The amount of gallic acid in the emblica extract was measured using HPLC as described from section. All determinations were performed in triplicate.

3.3.10 Clinical study

In this study various subjective and objective skin conditions were evaluated and the associations these measurements were examined for relationship of skin types.

For *in vivo* studies, a randomized, double blind clinical study was conducted to evaluate the safety and efficacy of 2 cosmetic creams for reducing hyperpigmentation and anti-ageing in human. The products in this study consist of the 1% emblica liposomes cream and liposomes cream. The protocol of this study is shown in Table 4.

Table 4 Experimental study

Number of volunteers	40 (n=20)
Age of volunteer	25 – 45 years old
Application	Apply on the face
Application dose	0.5 g/ (2 times per day)
Duration	1 month
Evaluation	According to prefixed time points
Parameters	Skin structure
	Skin elasticity
	Skin brightening
	Skin exfoliation
	Skin hydration

Appropriate subject selection remains one of the key for obtaining a successful clinical outcome of skin whitening. Most publications to date have reported

modest to moderate subjective and objective evaluations (Sasaki *et al*., 2007). The population in this study consisted of 40 (30 women and 10 men) with age ranging from 25-35 years old who were receiving no medication. Subjects who had extensive pigmentary disorders e.g. cholasma, ephelides, and multiple solar lentigines on the face were exclude from this study (Kawada *et al*., 2002). Prior to the study, the irritation test was monitored in all subjects by applying the 1% emblica liposomes cream and liposomes without emblica cream at the forearm for 100 mg formulation/5 cm^2 for 24 hours. The subjects who had irritated skin after apply the products were excluded from the study. Then, the subjects who have been passed the irritation test were allowed to apply the formulations on the face. The subjects were requested to apply the creams twice daily for 1 month. The evaluations were done before and after the treatment. During the study, recreational exposure to sunlight without sunscreens was inhibited. Efficacy was determined using an expert clinical evaluation, instrumental assessments, subject self-assessments and digital photography at baseline (week 0) (Sefton *et al*., 2000) and weeks 1, 2 and 4.

Testing procedure

The evaluation consisted of subject self-assessments, clinical evaluation and profilometric measurement from skin. Furthermore, the usages of creams were determined by weighting the jars before and after use. The subjects assessed the cosmetic properties of the cream i.e. texture, scent and absorption. On the basis of the findings obtained in these *in vitro* experiments, we designed the schedule of a series of *in vivo* experiments, carried out to evaluate the ability of cosmetic

products containing the 1% emblica liposomes to reduce wrinkles and hyperpigmentation in healthy human volunteers.

Inclusion criteria of this study consisted of skin tone of the subjects, not receive any dermatological treatments and the subjects who live in PSU or around because ease to follow up and exclusion criteria consisted of the subjects who had pigmentation disorder, allergy to any compositions in the creams, no participation in the whole experiments and the compliance of the subjects less than 20% of creams. Evaluation of clinical study consists of;

1. Skin structure to capture the texture of skin put the i-Scope on the left or right cheek that the illustration indicates above and adjusts the focus. Its shows the condition of skin and skin type the system displays fineness and orientation both of them were graded A; good, B; fair and C; poor.
2. Skin dullness was analyzed by Etude; skin counseling system put the i-Scope on the left or right cheek the system shows the transparency, dullness, degree of pigmentation e.g. mole and blemish the skin brightening was graded A; good, B; fair and C; poor.
3. Skin exfoliation monitored the keratin by attaching the B-squames sampling sticker on the face for 10 seconds and peeling its put the i-Scope on the left or right cheek. Skin exfoliation was graded A; balance, B; keratin retain more over and C; less of keratin.
4. Skin hydration put the Sensor on the left or right cheek to measure and the system shows the skin hydration. The simulation of sensor was graded low, middle and high.

5. Sebum was evaluated by attachment the Sensor onto the cheek. Make sure that the Sensor surface has been attached to skin of the entire Sensor's surface.
6. Skin elasticity put the sensor on the left or right cheek to measure and the system shows the skin elasticity. The simulation of sensor was graded low, middle and high.

Furthermore, the results should lead to an improved understanding of the emblica liposomes permeation and their effects on skin. Thus, the results will be useful in designing specific liposomes formulations for cosmetic application.

3.3.10.1 Sensory evaluation

At the end of the study, all subjects completed a questionnaire rating their degree of satisfaction with the emblica liposomes cream on five-point scale (excellent = 5, good = 4, fair = 3, poor = 2, very poor = 1).

3.3.11 Statistical analysis

The Statistic Package for Social Science (SPSS for windows) was used for data analysis. The data were analyzed and compared by t-test. The level of statistical significance was taken at p value of less than 0.05 levels.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation of crude extract

The extraction of emblica with 95% ethanol gave a dark-brown crude extract. The yield based on the dried extract was 35. 47%. The crude extract was pack in the tight container and kept in refrigerator.

4.2 Antioxidant activity of emblica extract

The antioxidant activity of emblica extract was determined by means of DPPH radical scavenging assay that is particularly suitable for evaluation of antioxidant activity of crude extract (Poli *et al*., 2003; Scartezzini *et al*., 2006) method of Williams *et al*. (1995) and ascorbic was tested as positive control. Briefly, mode of action of DPPH is a stable nitrogen-centered free radical, its color changes from violet to yellow when is reduced by either the process of hydrogen or electron donation. Because of performance this reaction can be considered as antioxidant (Rathore *et al*., 2011). The use of DPPH provides an easy and rapid way to determine the antioxidant activity in terms of EC_{50} (substrate concentration to produce 50% reduction of DPPH) (Molyneux, 2004).

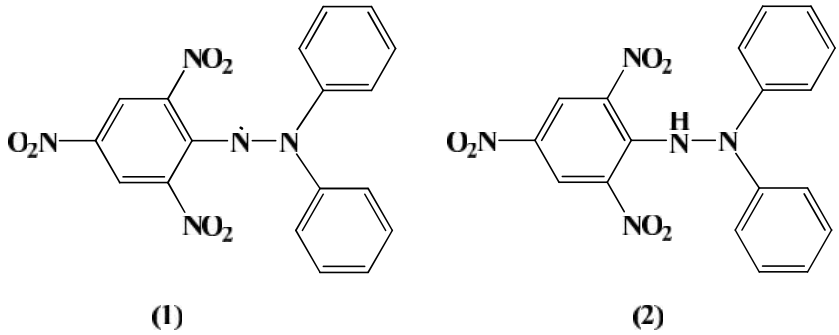


Figure 14 Structures of (1) 2,2-diphenylpicryl-1-hydrazyl and (2) its reduced form

Table 5 Comparative antioxidant activities of emblica extract and ascorbic acid

Sample	EC_{50} (µg/mL)
Emblica extract	2.47±0.02
Ascorbic acid	2.18±0.01

The values are Mean ± SD (n=3)

As shown in table 5 no significant difference was (p<0.05) found between the antioxidant activity of emblica extract and ascorbic acid by which results depended upon the presence of hydrolysable of tannins having ascorbic acid-like action have been reported (Naik *et al*., 2005; Khan, 2009). According to the results, it is evident that the interaction of potential antioxidant with DPPH associated with its structure as viewed in figure 14. Each sample was obtained by linear regression analysis of dose response curve plotting between %DPPH radical scavenging versus concentrations as well. This results supported the previously data that emblica is a promising source of potential antioxidant for cosmetic application. However, the antioxidant activity of the ethanolic extract of emblica reported in this study was lower than that reported by Nobnorb (2008) (1.41 ± 0.08 µg/mL) may due to long time storage resulted from EC_{50} value was increased whereas Lui *et al*. (2008)

reported that the EC_{50} of methanolic extracts of emblica fruits from six regions in China were in range of 11.23 ± 0.9 to 45.44 ± 1.0 µg/mL because of the variation in type and the extent of antioxidant phytocompounds obtained in different extract samples. Mahattanapokai (2003) used hexane, ethyl acetate and methanol as solvent extraction so reported the results of free radical scavenging assay in terms of EC_{50}. It was found that the ethyl acetate showed EC_{50} = 2.66 µg/mL. Similar to the study of Homklob *et al*. (2010) emblica fruits from four regions of Thailand for examples Prachuapkhirikhan, Kanchanaburi, Mahasarakham and Buriram were collected and ethyl acetate was used as the solvent extraction then the results showed that the EC_{50} varied between 0.025 - 0.037 mg/mL. In addition, Charoenteeraboon *et al*. (2010) reported the antioxidant activity of emblica extract in water extraction that was high 51.3 µg/mL. According to the previously reports it can be summarized that solvent extractions and the sources of emblica fruits largely influenced to the content of bioactive compounds (Liu *et al*., 2008). Besides, the antioxidant activity of tannin based emblica extracts was found to be 12 µg/mL (Chaudhuri *et al*., 2004). In contrast to the long-term belief, emblica fruits showed their potential because of ascorbic acid content, Ghosal *et al*. (1996) reported that emblica fruits did not contain ascorbic acid but also contain two hydrolysable tannins of low molecular weight which exhibited very strong antioxidant activity (Ghosal *et al*., 1996; Majeed *et al*., 2009).

4.3 Total phenolic content of emblica extract

Phenolic compounds are commonly found in both edible and inedible plants, they have been reported to have multiple biological effects, such as; antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to

their redox properties which can play an important role in adsorbing and neutralizing free radical, quenching singlet oxygen (Osawa, 1994; Kumaran and Karunakaran, 2006). This method also called gallic acid equivalence (GAE) method is the mixture of phosphomolybdate and phosphotungstane used for the colorimetric assay of phenolic antioxidants. It works by measuring the amount of substance being tested needed to inhibit the antioxidant of the reagent. Since plants produce an extraordinary diversity of phenolic metabolites that act as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the plant extracts (Ainsworth and Gillespie, 2007). Among the several assays available to quantify total polyphenolic, the Folin-Ciocalteu method is one of the most generally use. So, Folin-Ciocalteu reagent is commercially available for polyphenols quantification and was used in this study.

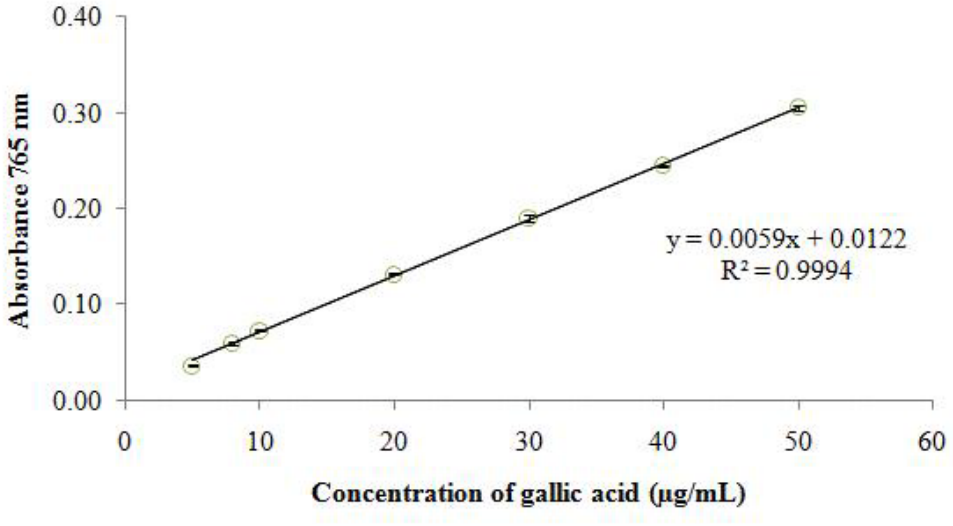


Figure 15 Calibration curve of gallic acid at UV absorbance 765 nm

The content of phenolic compounds in the ethanolic emblica extract was calculated from a regression equation of the calibration curve (y= 0.0059x + 0.0122, r^2 = 0.9994) and expressed as GAE. Total phenolics content was found to be 274.58 ± 0.007 mg GAE/g extract. This is lower than the previously value (358.92 ± 2.09 mg GAE/g extract) reported by Nobnorb (2008) and lower than value (290 ± 0.7 mg GAE/g extract) reported by Mayachiew and Devahastin (2008). Total phenolic content, on the other hand, was higher than 241 ± 8 mg GAE/g reported by Shukla *et al*. (2009) as well. According to Kumaran and Karunakaran (2007) that total phenols in *Phyllanthus* species were in range of 171-380 mg GAE/g extract. The mechanisms of phenolic compounds for antioxidant activity include inactivating lipid free radicals and preventing decomposition of hydroperoxides into free radicals. It can be observed that the content of phenolic in emblica extract correlates with the antioxidant activity. Some authors have been reported that there is no correlation between the content of these main antioxidant compounds and the radical scavenging capacity (Yu *et al*., 2002). For this purpose, Nobnorb (2008) carried out the correlation of phenolic content and antioxidant activity of emblica extract so the results showed high linear correlation coefficient (r^2 = 0.9762), recently. The results implied that phenolic compounds are likely to contribute to the radical scavenging activity of emblica extract (Kumar *et al*., 2006; Lou *et al*., 2009; Shukla *et al*., 2009).

The chemical and pharmaceutical investigations of emblica fruits have been well explored. As far as literature reports, several properties focusing on powerful antioxidant of emblica fruits was attributed to their ascorbic acid content (Kapoor, 1990); however, recently, a board spectrum properties have been found and isolated from emblica fruits such as polyphenols compounds (Ghosal *et al*., 1996;

Bhattacharya *et al*, 2000; Kumar *et al*., 2005; Kumaran and Karunakaran, 2006; Raghu *et al*., 2007; Liu *et al*., 2008; Lou *et al*., 2009; Majeed *et al*., 2009; Madhavi *et al*., 2010; Sawant *et al*., 2010). From this point of view, emblica fruits have been the subjects of study by several researchers. Although there have been different approaches to their analysis, the separation and quantification of the phenolic compounds of a plant extract remains difficult, especially for simultaneous determination of different groups of phenolic in a sole analysis (Govindarajan *et al*., 2007). HPLC-DAD chromatographic method seems a suitable for separation and quantification of phenolic compounds in plant extracts (Merken and Beecher, 2000). It is essential to examine of an optimized, validated HPLC method for the standardization then apply the method in real sample analysis using gallic acid as marker, however.

4.4 Validation of gallic acid assay

Method validation is a process used to confirm that an analytical procedure employed for a specific test is suitable for the intended use. The method was validated according to ICH guideline for linearity, precision and accuracy (Lister, 2005).

4.4.1 Linearity

The calibration curves for gallic acid fitted by plotting area versus concentration were linear in the range 10 - 50 µg/mL (five concentrations). A standard curve of gallic acid is shown in figure 15. The typical regression equation were y = 180.3208x – 301.6103 and the correlation coefficients (r^2) were higher than 0.999 (n=5) confirming the linearity of the method.

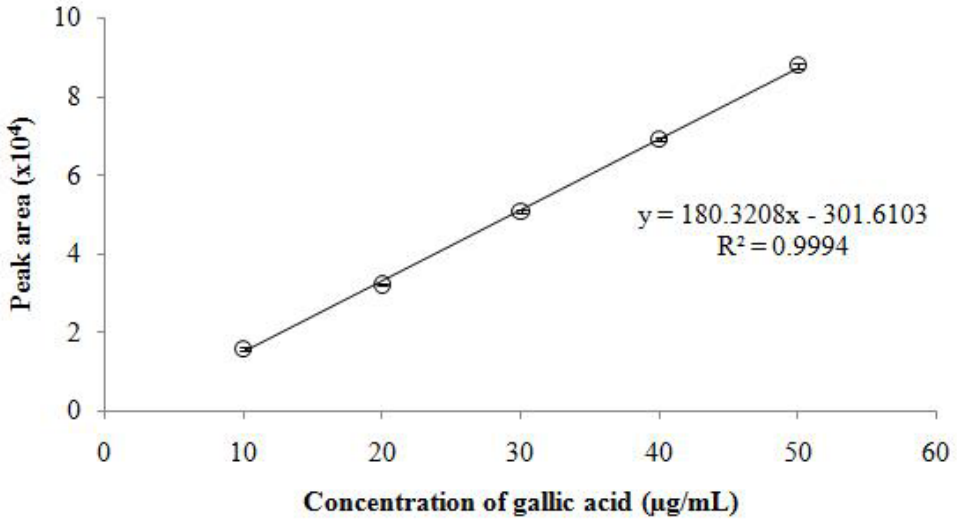


Figure 16 A standard calibration curve of gallic acid. The plotted data are mean ± SD (n=5). Solid line is linear regression fit to the data.

4.4.2 Accuracy and precision

The accuracy and precision of the method were determined by analyzing the percentage of recovery from intra- and inter-day variability of the essay. Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between an accepted reference value and the value found in the sample whereas precision is usually measured as a standard deviation of a set of data. Gallic acid was spiked in three different concentrations at 15, 25 and 45 µg/mL. According to ICH guidelines accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels. Table 6 shows the intra-day reproducibility of gallic acid assay.

Table 6 Intra-day variability of gallic acid analysis

Concentration (µg/mL)	**Day**	***Mean of measured conc. (µg/mL)**	**Average Recovery (%)**	**%RSD**
15	1	15.27 ± 0.16	101.81	1.44
	2	15.03 ± 0.11	99.41	1.07
	3	15.29 ± 0.28	100.11	1.87
25	1	25.35 ± 0.14	101.39	1.96
	2	25.04 ± 0.29	99.69	1.50
	3	25.20 ± 0.11	100.54	1.08
45	1	46.37 ± 0.27	102.69	1.01
	2	44.37 ± 0.10	99.89	0.63
	3	44.66 ± 0.30	101.32	1.09

*n=3

In table 6 data are shown relative to 100%, the accuracy of the average measured concentration for each day, reported in term of percent recovery is between 99.41 - 102.69%. The intra-day precision are varies range from 0.63 to 1.96 as well. In this study, both the accuracy and precision pass the acceptance criteria of 98 - 102% and 2%, respectively (Swartz, 2010).

Table 7 Inter-day variability of gallic acid analysis

Concentration (µg/mL)	***Mean of measured conc. (µg/mL)**	**Average Recovery (%)**	**%RSD**
15	15.20 ± 0.14	100.44	1.97
25	25.20 ± 0.15	100.54	1.53
45	45.13 ± 1.08	101.42	2.20

*n=9

Table 7 shows that the relative standard deviation varies between 1.97 to 2.20% and the recovery is in the range of 100.44 - 101.42% that obtained from concentration values minimum, medium and high. The results of validation gave the good repeatability and inter-day precision, lower than 2%. These results indicated that the assay is accurate and reproducible enough for its application (ICH). Therefore, the results obtained from HPLC method can be regarded as selective, accurate and precise for the quantification of gallic acid.

4.5 Quantitative of gallic acid of emblica extract

Standard solutions of gallic acid, in the 1 - 50 µg/mL concentration range, were prepared and injected to HPLC system.

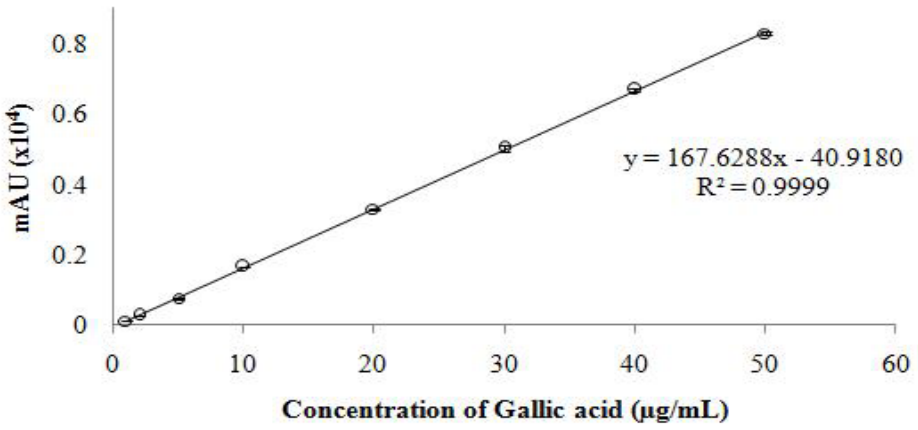


Figure 17 Standard calibration curve of gallic acid

A good linearity obtained from calibration curve was found from 1 - 50 µg/mL gallic acid, as shown in figure 17 and the linear regression equation was y = 167.7941x - 38.9903 (r^2= 0.9999), where y is peak height and x is the gallic acid concentration, expressed as µg/mL. Since emblica fruits contain largely amount of tannins such as, ellagic acid and gallic acid, it is essentially required to maintain their quality and purity for safety and efficacy. In this study, the amount of gallic acid in the ethanolic extract of emblica was measured using the previously published method of Kumaran and Karunakaran (2006) with minor modification.

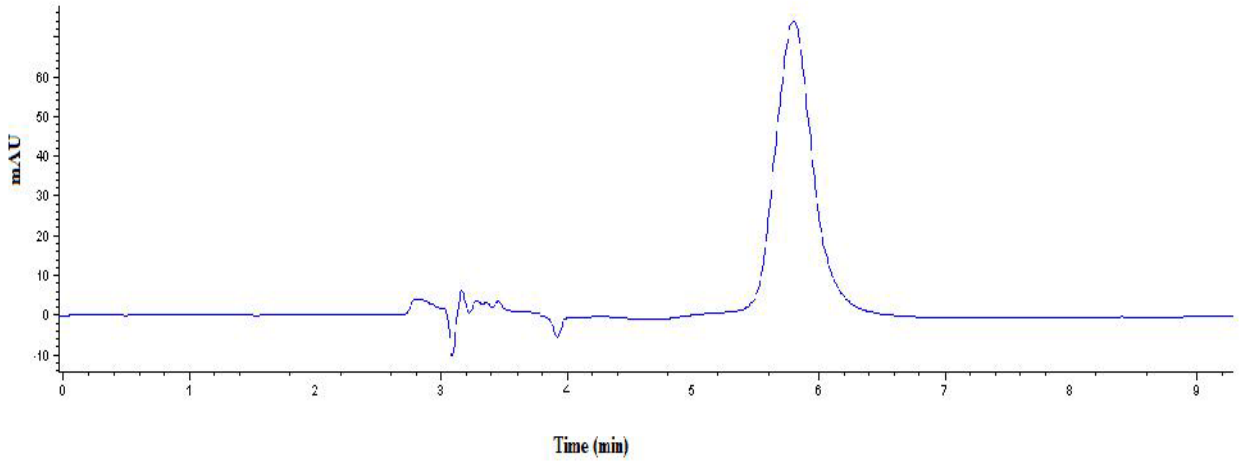


Figure 18 Chromatogram of standard gallic acid in methanol

The retention time of gallic acid was 5.7 minutes (peak). These data was confirmed by comparison to the spectra obtained from the authentic standard of gallic acid as shown in figure 18.

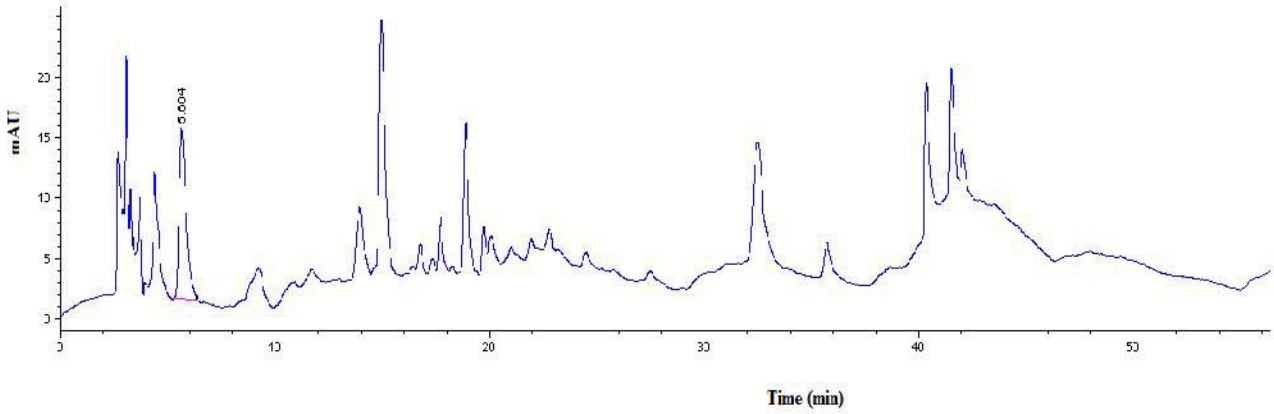


Figure 19 Chromatogram of emblica extract dissolved in methanol

The amount of gallic acid in emblica extract was found to be 34.27 ± 0.67 mg/g. This quantification is lower than the value (71.89 ± 6.58 mg/g) obtained from ethyl acetate extraction reported by Kumaran and Karunakaran (2006) and higher than the value (19.12 mg/g) reported by Lou *et al*. (2009). These data support the presence of gallic acid that previously reported by several publishing (Kumaran and Karunakaran, 2006; Kumar *et al*., 2006; Lou *et al*., 2009; Majeed *et al*., 2009). In contrast to previous authors Mayachiew and Devahastin (2008) asserted that hydrolysable tannins were the main compounds because these compounds have high polarity due to the presence of hydroxyl group. In this study, it might be possible to conclude that gallic acid and other phenolic compounds contributed to the antioxidant activities of emblica fruits extract.

4.6 Preparation of the emblica liposome

Liposomes containing emblica extract was typically prepared by modified ethanol injection technique using 1 % emblica extract. The composition of emblica liposomes included SPC: Tween 80: Deoxycholic acid (DA) with the ratio of 84: 16: 2.5 (by weight) and the total lipid concentration of 200 µmol/mL. This emblica liposomes formulation gave yellowish colloidal appearance with particle size and entrapment efficiency is summarized in the table 8.

Table 8 Composition of emblica extract liposomes and the physical appearances

Compositions	**Total lipid (µmol/mL)**	**Ratio**	**Particle size (nm) (n=3)**	**% Entrapment Efficiency (n=3)**
SPC: Tween 80: DA	200	84: 16: 2.5[a]	247.7 ± 5.49	46.79 ± 0.21

[a] weight ratio

In view of merits of liposomes in skin topical application and their influencing factors, the parameters of liposomal preparations could be achieved, for examples; compositions, additives and their characteristics. Firstly, the composition of liposomes, in this study phospholipid which forms flat lamellar sheet used the SPC as primary materials for vesicular delivery systems due to natural origin and similarity to the lipid in the skin. The fundamental property of phospholipids, they thus become the external wall of water trapping vesicles, which are themselves suspended in the water (Benita *et al*., 2006; Liu and Hu, 2007). Moreover, some additives are candidates such as surfactants, buffer, surface charges (Kozubek *et al*., 2000; Manosroi *et al*., 2002) and preservative. DA was used as anionic surface charge on vesicles, thus giving them in terms of stability for their aggregation and fusion. They may also cause an increase in the interlamellar space resulting in a greater capacity for the encapsulation of certain active substances (Kozubek *et al*., 2000; Sonico *et al*., 2005; Benita *et al*., 2006; Patravale and Mandawgade, 2008). Ogiso *et al*. (2001) reported that the negative charge improves skin permeation of liposomes. In addition, surfactants Tween 80 tend to fluidize membranes and also make liposomes very elastic, resulting in highly deformable vesicles (Walters *et al*., 1993; Cevec *et al*., 1996; El Maghraby *et al*., 2000; El Maghraby *et al*., 2004; Braun *et al*., 2006; El Maghraby *et al*., 2008). These elastic aggregates resulting from surfactants and SPC resemble in liposomes in

morphology but not in function (Trotta *et al*., 2001). Buffer, in short, pH 5.5 was used as water vehicles because of good stability of emblica extract and closely to the pH of skin point of view (Nobnorb, 2008).

Methods of preparation of liposomes and their potential in topical application especially for cosmetic and dermatology have been purposed (Egbaria and Weiner, 1990). Typically emblica liposomes were prepared by means of modified ethanol injection method and the particle size 247.7 ± 5.49 nm as a result of method preparation; it can be concluded that this procedure gave large unilamellar vesicle (LUV) liposomes (Kulkarni, 2005). Both particle size and the content of active encapsulated inside liposomes are an important parameter, particularly in cosmetic applications (Verma *et al*., 2003b; Saraf, 2010; Contri *et al*., 2011). The encapsulation efficiency varies depending on the method preparation, type of lipid and active itself. Capture-volume capacity of liposomes is measured by calculating for total lipid of 200 μmol mL^{-1} unit that is affected to both entrapment efficiency and vesicular size. It is essential to evaluate the entrapment efficiency. Among several methods in order to evaluate efficiency of drug encapsulation, separation techniques have been introduced for measure that is suitable (Lasic, 1998; Bergmann *et al*., 2011). Not only exhaustive dialysis, filtration devices but also gel exclusion column chromatography would be promised for small molecular weight drugs, on the other hand; separation by centrifugation is supposedly free from the risks of external medium dilution (Margalit and Yerushalmi, 2006). Most of the polyphenols present in emblica fruit are phenolic acid, commonly known as gallic acid which is hydrophilic property and also polarity that was entrapped in the aqueous inner core of liposomes. For instance, epigallocatechin-3-gallate is the ester of gallic acid and also known as natural

antioxidant in tea. Fang *et al*. (2006), Fang and Bhandari (2010) reported the encapsulation of catechin and epicatechin that located within the bilayers thus increasing the entrapment. Though the entrapment efficiency of emblica liposomes used in this study about 46.79% was lower than 84.27% (Nobnorb, 2008) that used dialysi technique and measured by UV absorbance expression as GAE. It is not surprisingly with differential entrapment efficiency of emblica liposomes because the procedures and technical assay were used. In theoretical encapsulation efficiencies of above 70% are never resembled in experimentally, where 50% are considered to be high and generally 20-35% that can be obtained (Lasic, 1998).

4.7 Formulation of cream bases

A variety of skin care products exist in marketplace. They fulfill a variety of functions by either acting directly on the skin or being a cosmetically elegant vehicle for the delivery of specific active ingredients (Epstein and Simion, 2001). In skin care and cosmetic products, liposomes are formulated in an appropriate matrix; for examples, serums, lotions, gels or creams. Emulsion systems are utilized widely in the formulation of topical pharmaceutical and cosmetic preparations (Tadros, 1992; Magdassi, 1997; Soriano *et al*., 2001; Walters and Brain, 2004; Smith *et al*., 2005; Somasundaran *et al*., 2007; Lu and Gao, 2010). Ericsson *et al*. (1991) reported that lamellar structure has been utilized in emulsion to help in stabilizing; therefore, o/w emulsion (Allen, 1998; Mollet and Grubenmann, 2001; Gupta, 2003; Padilla *et al*., 2005; Wille, 2005; Bunjes and Siekmann, 2006; Baroli, 2010) was selected to incorporate liposomes because of the pleasant skin sensory perception

(Draelos, 2005; Otto *et al*., 2009; Appa, 2010). In fact, a very lipophilic molecule will easy partition in stratum corneum, will leave it difficulty, on the other hand; a hydrophilic molecule will be poorly penetration. Ingredients with the following substances of cosmetic grade are shown in table 3; all cream bases were delicately prepared by two phase heat system. Physical characteristics of cream bases are presented in table 9. All formulations had good appearance with white color. The texture of them was homogenously. It is well established that liposomes comprising of phospholipids are not stable in the presence surfactants, therefore, it is challenging to formulate liposomes in personal care products since surfactants are commonly used in cosmetic (Nomura *et al*., 2001; Kulkarni, 2005).

Table 9 Physical properties of cream base before and after heating-cooling test (6 cycles)

Formulations	Physical appearances	pH		Viscosity (cps)	
		Before FT	After FT	Before FT	After FT
Rx 1	White viscous cream	6.92 ± 0.03	6.87 ± 0.03	54542 ± 213	52261 ± 122
Rx 2	White viscous cream	6.88 ± 0.01	6.77 ± 0.02	45377 ± 58	44276 ± 110
Rx 3	White low viscous cream	5.47 ± 0.04	5.35 ± 0.03	78509 ± 65	77403 ± 334
Rx 4	White viscous cream	5.64 ± 0.03	5.60 ± 0.04	49368 ± 265	48329 ± 112

FT = Freeze-thaw cycle

The values are Mean ± SD (n=3)

Table 10 Evaluation of physical characteristics of cream base

Physical characteristics	Formulation			
	Rx1	Rx2	Rx3	Rx4
Spreadability	3	3	3	4
Smoothness	4	3	2	4
Viscosity	4	4	4	4

1 = little 2 = moderate 3 = much 4 = very much

Cream bases Rx 4 was also selected for developing cream containing emblica extract because of good physical appearance in color and smoothness. Moreover, it was physically stable after freeze-thaw cycle (6 cycles). In this study, emblica liposomes were incorporated into cream base 1 % w/w based on EC_{50} value. Both ethanolic emblica extract and blank liposomes were also incorporated at concentration 1 % w/w for use as control. Freshly prepared emblica extract had brownish yellow color. The tone of color depended on the concentration of the emblica extract in the formulation. The color of 1% emblica extract cream, however; was acceptable as skin creams. Not only the physical appearances (color, smoothness, oily, and phase separation) but also antioxidant activity was examined. Moreover, pH, viscosity of the cream formulations was measured before and after heating-cooling cycle test. It has been found that all of creams seemed to be stabilization in the protocol of the study. In addition, the chemical in terms of antioxidant activity of creams was evaluated before and after heating-cooling test as well.

Table 11 Physical properties of formulations before and after heating-cooling test

Formulations	Appearances	pH		Viscosity (cps)	
		Before FT	After FT	Before FT	After FT
Rx4-1	White viscous cream	5.42 ± 0.02	5.41 ± 0.03	43484 ± 109	43189 ± 185
Rx4-2	White viscous cream	5.64 ± 0.03	5.63 ± 0.02	48094 ± 47	48165 ± 234
Rx4-3	Brown viscous cream	4.32 ± 0.03	4.22 ± 0.02	19030 ± 30	18815 ± 42
Rx4-4	White viscous cream	5.67 ± 0.03	5.54 ± 0.04	49192 ± 124	48880 ± 109

Physical properties of cream Rx 4-1; 1% Emblica liposomes cream, Rx 4-2 Liposomes cream, Rx 4-3 1% Emblica cream and Rx 4-4 Cream base.

4.8 *In vitro* skin permeation study

In vitro data is more widely accepted for use to determine the suitability of a compound or product for use in clinical studies (Walters *et al*., 1993; Brain *et al*., 2002; Shah, 2005; Tonucci, 2005). In order to evaluate the potential of emblica liposomes as a novel encapsulation and transport system into the skin was examined. For this reason, the *in vitro* skin permeation study was performed using modified Franz diffusion cells and pig skin was used as model for prediction of *in vivo* situation (COLIPA, 1997). In short, the experiments were performed by applying consisted of 1% emblica liposome cream, 1% emblica extract cream, liposome cream, conventional cream, 1% emblica liposome and blank liposomes as well. The result is shown in figure 20; in this study, 1% emblica liposomes cream, 1% extract cream and emblica liposomes were also calculated the gallic acid in the receptor compartment by HPLC method. No gallic acid was detected in the receptor compartment following the application all formulations containing emblica fruits extract. The results indicated that liposomes are not beneficial in delivery of emblica fruits extract through systemic compartment. There is evidence to show that the residual amount of gallic acid might be retained in the skin after administration (Trotta *et al*., 2001).

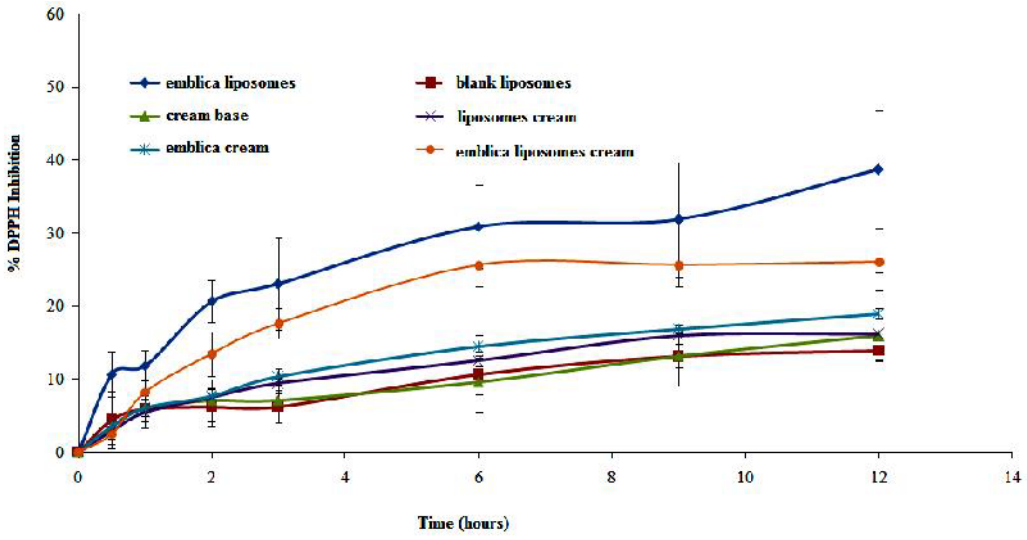


Figure 20 Antioxidant activity of emblica extract delivered from cosmetic formulations. The plotted data are Mean ± SD (n=3)

There is no gallic acid from receptor fluids detection in HPLC system, on the other hand; indirectly method such as DPPH radical scavenging assay in terms of antioxidant activity could be observed. It is obviously seen from the figure 20 that, 1% emblica liposomes cream was highest of all formulations more than the emblica liposomes, respectively. Despite research and high interest from cosmetic, the use of liposomes is suitably for topical products that are used on the skin meant to stay on the stratum corneum and not penetrate into the dermis (Tonucci, 2005). In accordance with mode of actions of liposomes has been reported (Kirjavainen *et al*., 1996), two main reasons may be hypothesized to completely explain the effect of phosphatidylcholine and emblica fruits extract on the penetration of liposomes into the skin.

In this study, it can be summarized that liposomes could be enhanced the emblica fruits extract as active compound in the skin, suggesting that liposomes

are only useful for topical dermal delivery (Shigeta *et al*., 2004; Nguyen and Bouwstra, 2006; Cho *et al*., 2007). Form this results related to the study of Fang *et al*. (2006) that gallic acid ester as epigallocatechin-3-gallate strongly interact within the bilayers due to its high level of encapsulation, it was difficult for gallic acid ester to penetrate from the vesicles; therefore it was not permeated through receptor compartments. However, the molecular weight of epigallocatechin-3-gallate was 458.4 Dalton (USP) for this reasons, it is difficult to penetrate across skin because of the properties of stratum corneum. This result can be implied that gallic acid was not penetrated through the skin.

4.9 Analysis of gallic acid retained on the skin

Besides the assay of the active drugs in the receptor compartment, the amount of the substance retained on the skin is also commonly evaluated using diffusion cells (Contri *et al*., 2011). Among the techniques employed to determine active concentrations in skin, the excision of skin tissue that can be effectively assessed is the most invasive but still practicable (Surber *et al*., 2005; Walters and Brain, 2004) use in this study. Briefly at the end of the *in vitro* experiments, pig skin was extracted and analyzed for its amount by HPLC. In order to determine the extraction recovery from the skin is usually performed where excised skin is spiked with gallic acid. Methanol was used as the solvent for extraction of gallic acid from skin because of its high recovery value determined from gallic acid spiked in the blank skin (92.60–106.68%) (Diembeck *et al*., 1999; SCCNFP, 1999). In this experiment, the formulations containing emblica extract consisted of emblica liposomes, 1% emblica extract cream and 1% emblica liposomes cream as well was

performed in order to calculate the gallic acid retaining in the skin. *In vitro* study, amount of gallic acid accumulated in the skin after 12 hours permeation study was expressed in figure 21, 1% emblica liposomes shown the highest gallic acid accumulated in skin 14.90 ± 5.15 µg/cm^2, 1% emblica extract cream shown gallic acid 4.82 ± 0.15 µg/cm^2 and 1% emblica liposomes cream shown the lowest of gallic acid 1.18 ± 0.20 µg/cm^2 as well the results indicated that liposomes carrier could provide a mode for delivery of gallic acid. Liposomes are known as penetration enhancer, this property is usually associated with polar head groups of liposomal bilayers (Lauer, 2005). Liposomes are the vesicles that transport cosmetic agents into the horny layer (Lautenschlager, 2001; Essa *et al*., 2005).

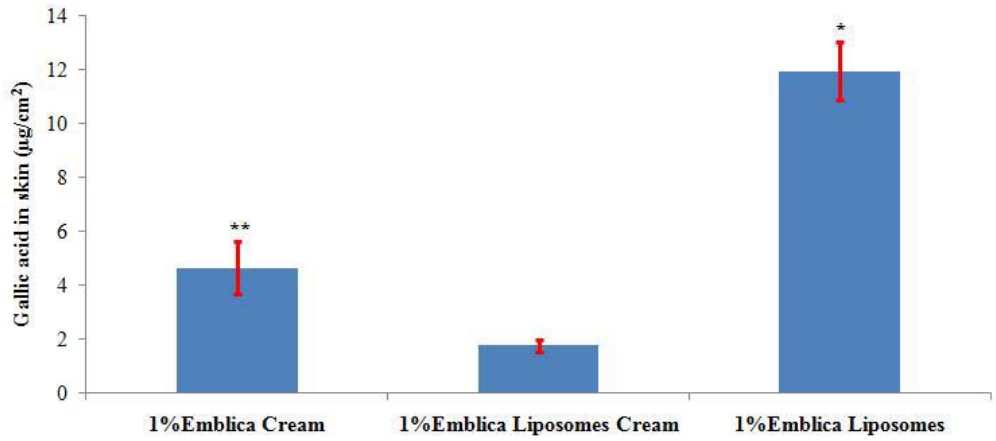


Figure 21 Amount of gallic acid accumulated in skin Mean ± SD (n=3)

(*p<0.05, 1% emblica liposomes vs. all formulations)

(**p<0.05, 1% emblica cream vs. 1% emblica liposomes cream)

Since the main barrier of the skin is located within the stratum corneum that is considered as the target site for cosmetic (Barrett, 1969; Tadros, 1992; Turner and Nonato, 1997; Arct *et al*., 2002; Roberts and Cross, 2002; Trotta *et*

al., 2002; Betz *et al*., 2005; Choi and Maibach, 2005; Muller *et al*., 2005; Tonucci, 2005; Benita *et al*., 2006; Esposito *et al*., 2007; Remon, 2007; Tadros, 2008; Baumann and Saghari, 2009; Förster *et al*., 2009). Moreover, it is well known that the skin may act as a negatively charged membrane (Burnette and Ongipattanakul, 1987; Montenegro *et al*., 1996; Sinico *et al*., 2005; Godin and Touitou, 2007) and it has been generally reported that the presence of any charges on the surface affects the drug diffusion through the skin. For this reason, negatively charge can improve drug accumulation in the superficial skin strata (Sonico *et al*., 2005). This is an important feature, as the novel carrier can retain or even sustain the release of these active substances in the epidermis (Egbaria and Weiner, 1990; van Kuijk-Meuwissen *et al*., 1997). Interestingly, liposomes are of little value as carriers for drug delivery because they neither invade nor deeply penetrate the skin (Tadros, 1992; Kirjavainen *et al*., 1996; Dayan and Touitou, 2000; Wester and Maibach, 2001; Trotta *et al*., 2002; Richert *et al*., 2003; Verma *et al*., 2003ab; Honeywell-Nguyen *et al*., 2004; Benson, 2005; Dayan, 2005; Muller *et al*., 2005; Roberts *et al*., 2005; Dayan, 2006; Fang *et al*., 2006; Honeywell-Nguyen and Bouwstra, 2006; Elsayed *et al*., 2007; Dayan *et al*., 2007; Blume, 2008; Fairhurst and Loxley, 2008; Jesorka and Orwar, 2008; Förster *et al*., 2009; Baroli, 2010; Santana and Zanchetta, 2011). The potential of liposomes for topical uses was first introduced by Mezei and Gulasekharam (1980) after that, many researchers have been developed of novel lipid vesicles as carried for the delivery of drugs to skin. A board spectrum of application of liposomes on the skin has been reported in route of administration such as topical skin products, transdermal or percutaneous absorption of the vesicles. As results of controversy use of different drugs have led on remarkable actual capability of conventional liposomes which act

as topical product carriers (Santana and Zanchetta, 2011). A wide variation of lipids and surfactants are widely used in cosmetic preparations, most commonly the vesicles are composed of phospholipids or surfactants (Meizei and Gulasekharam, 1980, Planas *et al*., 1992; Walters *et al*., 1993; Wen *et al*., 2006). Elsayed *et al*. (2007) criticized in the potential of conventional liposomes as carriers for transdermal delivery that they fail to deeply penetrate the skin; on the other hand, liposomes remain confined to the upper layer of the skin so the question of penetration into the skin is still partially controversially discussed. At least it can be summarized that not any liposomes will penetrate; it seems to depend on its size and compositions (Muller *et al*., 2005), however. It is important for topically delivered emblica fruits extract to penetrate through the stratum corneum and to maintain an effective concentration near the melanocytes. In the point of view, present results provide a good rationale in the development of skin whitening and promising effectively in clinical trials. Liposomes technology offers the great opportunities for several new cosmetic products. Based on previous *in vitro* study results can be led to an improved understanding of liposomes penetration and their effects on skin (Richert *et al*., 2003). Thus, the results will be useful in designing natural antioxidant liposome formulations for cosmetic use with the aim to promote skin effects. Taking this point to consideration, liposomes combined with natural powerful antioxidant for restoration the barrier function and to provide an active delivery system at the same time. The composition and properties of emblica liposomes in the typically creams play an important role in their interaction with possible penetration of skin resulting in beneficial effects.

4.10 The *in vivo* evaluation of emblica liposomes cream

A key aspect of any cosmetic products is its safety and efficacy as demonstrated in controlled clinical trials. The clinical evaluation efficiency of 1% emblica liposomes cream was performed in a randomized double blind trial using 1% liposomes cream as a control. In this study, forty subjects aged between 25-45 participated and were divided in two groups with twenty subjects in each group. All subjects received emblica liposomes cream or liposomes cream, respectively for one month. Each formulation was applied on the face for two times. Skin conditions of subjects were record in the first time application. All data were collected and analyzed by skin counseling system.

In the period of this study, one subject was excluded because of hypersensitivity. Skin conditions comparing with 1% emblica liposome cream and 1% liposome cream were measured at week 0 (base line), 1, 2 and 4 by skin counseling system. For this reason, the statistically data was tested by means of student pair t-test. The *p*-value of less than 0.05 ($p<0.05$) was considerably studied to be statistically significant difference.

4.10.1 Skin texture

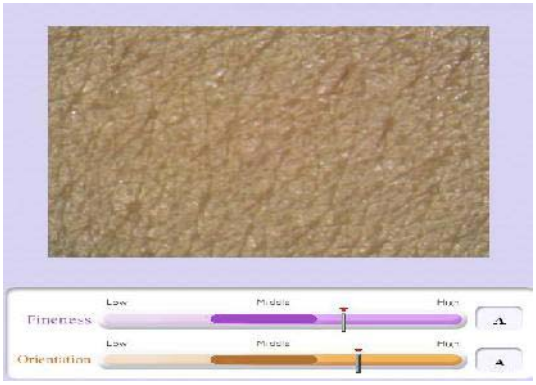
Table 12 Significant difference of skin texture between 1% emblica liposomes cream and 1% liposomes cream

Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	26.80	5.10	NS
	Liposomes cream	20	10.37	2.32	
1	1% Emblica liposomes cream	20	18.50	9.97	NS
	Liposomes cream	20	22.45	19.18	
2	1% Emblica liposomes cream	20	30.60	17.20	NS
	Liposomes cream	20	34.45	24.97	
4	1% Emblica liposomes cream	19	25.50	15.14	NS
	Liposomes cream	19	34.06	20.96	

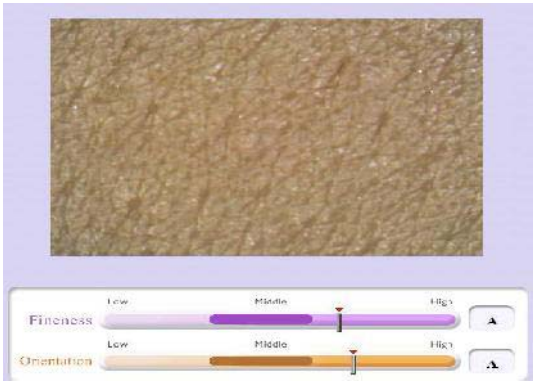
NS Non significance

S* Significance at $p < 0.05$

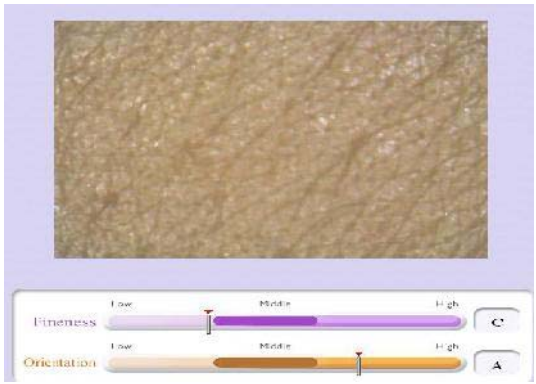
Skin texture is the unevenness or smoothness of the skin that even in peaks and valleys. Evaluation of the texture of skin plays a very important role in skin counseling. There were no significant different in skin texture improvement both in 1% emblica liposomes cream and liposomes cream. Figure 22 shows the skin texture taken from the cheek of subjects.



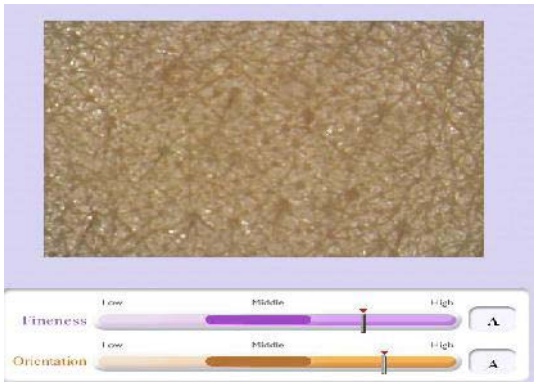
week 0



week 1



week 2



week 4

Figure 22 Skin texture and orientation

A = very good, B = good and C = poor

4.10.2 Skin orientation

Table 13 significant differences of skin orientation between 1% emblica liposomes cream and 1% liposomes cream

Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	77.05	14.12	NS
	Liposomes cream	20	75.75	17.27	
1	1% Emblica liposomes cream	20	77.10	8.18	NS
	Liposomes cream	20	77.00	14.40	
2	1% Emblica liposomes cream	20	76.50	13.59	NS
	Liposomes cream	20	77.20	21.14	
4	1% Emblica liposomes cream	19	81.39	9.62	S*
	Liposomes cream	19	72.11	15.93	

NS Non significance

S* Significance at $p < 0.05$

The skin orientation is achieved by light image based skin analysis. A digital camera combined with different lighting techniques can be used to visualize and subsequently analyze either a local area or the entire face for a number of feathers. The basically texture measurement is accomplished with white light. Evaluation the surface pattern of the peaks and valleys dividing the skin into tiny segments, homogeneity of the skin surface pattern can be determined. The results of 1% emblica liposome cream and 1% liposome cream were illustrated in table 13 above. There were significant differences in skin orientation in week 4.

4.10.3 Skin dullness

Table 14 Significant difference of skin dullness between 1% emblica liposomes cream and 1% liposomes cream

Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	48.80	19.14	NS
	Liposomes cream	20	55.25	22.11	
1	1% Emblica liposomes cream	20	57.20	23.17	NS
	Liposomes cream	20	61.45	14.69	
2	1% Emblica liposomes cream	20	41.85	28.14	S*
	Liposomes cream	20	67.45	18.56	
4	1% Emblica liposomes cream	19	52.21	26.19	S*
	Liposomes cream	19	74.21	14.44	

NS Non significance

S* Significance at $p < 0.05$

Skin dullness reported in terms of transparency at the same time therefore the degree of pigmentation was shown with skin dullness (Hermanns *et al*., 2000). The influence of the formulation is importance. The o/w emulsion containing 1% emblica liposomes was suited for obtaining an immediate moisturizing resulting from liposomes. Figure 23 shows the mean hyperpigmentation are fraction for subjects. In week 2, the skin dullness was significantly difference in subjects compared to the control respectively. Moreover, the transparency of subjects was continuously improved until finished experiment. As shown in figure 23 shows the dullness of subject skin, in the beginning dullness was grade B. Then the dullness was

clearly changed in week 2 being A, therefore; it can be concluded that emblica liposomes is the good promising whitening agent and safety for topical use.

4.10.4 Degree of pigmentation

Table 15 Significant difference of skin orientation between 1% emblica liposomes cream and 1% liposomes cream

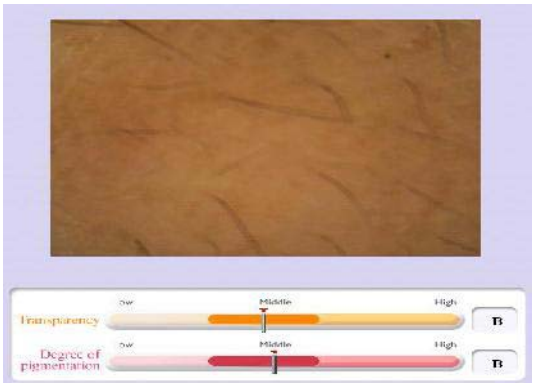
Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	77.05	14.12	NS
	Liposomes cream	20	75.75	17.27	
1	1% Emblica liposomes cream	20	77.10	8.18	NS
	Liposomes cream	20	77.00	14.40	
2	1% Emblica liposomes cream	20	76.50	13.59	NS
	Liposomes cream	20	77.20	21.14	
4	1% Emblica liposomes cream	19	72.11	15.93	S*
	Liposomes cream	19	81.39	9.62	

NS Non significance

S* Significance at $p < 0.05$

Good pigmentation models are considered the basis for such kind of whitening efficacy evaluation (Tain *et al*., 2009). As a result of skin dullness influenced by emblica liposomes was related to degree of pigmentation. Pigmentation or melanin index of the skin can be influenced by external factor like free radicals and UV light, causing sun damage and spot, which can accelerate the normal ageing process. This can influence the degree and distribution of melanin in the skin resulting in uneven pigmentation, for examples, dark spots and blemish. The results of degree

of pigmentation were illustrated in table 15 that shows the average of degree of pigmentation slightly increase no different significance. After use, in the week 4 degree of pigmentation was significantly increases. This result indicated that colour associated with melanin index as shown in figure 23 represents the degree of pigmentation in the subjects; in week 4 the result increased to A indicate that the color of skin was higher than baseline.



week 0

week 1

week 2

week 4

Figure 23 Skin dullness and degree of pigmentation

A = very good, B = good and C = poor

4.10.5 Keratinization

Table 16 Significant difference of keratin between 1% emblica liposomes cream and 1% liposomes cream

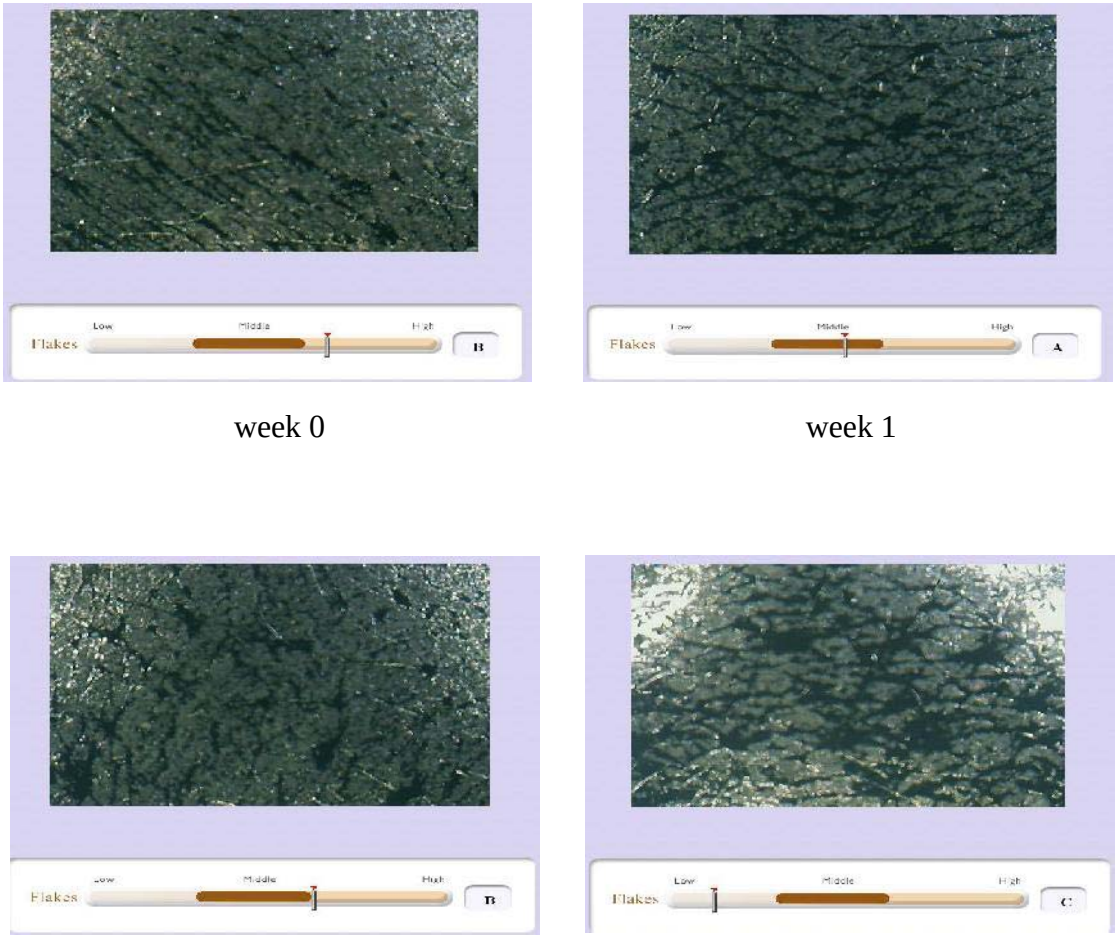
Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	38.65	28.75	NS
	Liposomes cream	20	51.25	28.82	
1	1% Emblica liposomes cream	20	51.00	34.62	NS
	Liposomes cream	20	50.35	29.16	
2	1% Emblica liposomes cream	20	48.35	26.72	NS
	Liposomes cream	20	49.80	31.89	
4	1% Emblica liposomes cream	18	43.44	22.34	NS
	Liposomes cream	18	39.88	31.03	

NS Non significance

S* Significance at $p < 0.05$

In general, components of the barrier, corneocytes and the intercellular lipid bilayers are constantly synthesized and secreted by the keratinocytes during the process of terminal differentiation (Pillai *et al*., 2010). The skin's corneal layers have a naturally consistent cell turnover cycle approximately 28 days (Fylnn, 2002). The turnover rate as well as size and shape of keratin cell are an indicator of the skin's flakiness and condition. As a role of skin desquamation it may be concluded that the hydrolysis of cholesterol sulfate in the stratum corneum plays a key importance for corneocyte removal (Madison, 2003). Moreover, excess cholesterol sulfate has been considered to alter the structure and function of the lipid bilayers (Zettersten *et al*., 1998; Bouwstra *et al*., 1999; Madison, 2003; Bouwstra *et al*., 2008). Removal of dead

cell on the top of skin is evaluated by means of an adhesive tape based on size, shape, and number of dead cells. This physical method provides a sign to binding forces that hold the corneocyte together. The efficacy of treatment with skin moisturizers or emollients that improve skin hydration and reduce scaling can be measured using this method (Pillai *et al.*, 2010).



week 0 week 1

week 2 week 4

Figure 24 Skin keratinization

A = Balance, B = More remnant and C = Less remnant

4.10.6 Sebum

Table 17 Significant difference of skin sebum between 1% emblica liposomes cream and 1% liposomes cream

Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	26.90	8.04	NS
	Liposomes cream	20	30.45	8.77	
1	1% Emblica liposomes cream	20	28.90	17.58	NS
	Liposomes cream	20	37.05	15.14	
2	1% Emblica liposomes cream	20	27.20	15.49	S*
	Liposomes cream	20	37.45	15.70	
4	1% Emblica liposomes cream	18	24.06	14.28	NS
	Liposomes cream	18	31.61	10.31	

NS Non significance

S* Significance at $p < 0.05$

Sebum is the mixture of fatty acids, triglycerides, proteins and other molecules produced by the sebaceous glands of the dermis. Sealing skin moisture in the corneal layer and preventing evaporation, sebum keeps hair and skin smooth and flexible. Inordinate sebum production can cause clogged pores possibly resulting in blemishes. Sebum is determined by Sensor that are important devices in esthetic skin counseling. Since Sensor give fast, relevant results for the key factors determining the actual skin condition. The results of 1% emblica liposome cream and 1% liposome cream were illustrated in table 17. There was no significant difference of sebum for subject in week 0 and week 1and week 4. However, it found that in week 2 the

difference of mean sebum was observed due to the condition of evaluation and skin condition of subjects.

4.10.7 Moisture

Table 18 Significant difference of skin moisture between 1% emblica liposome cream and 1% liposome cream

Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	26.70	8.51	NS
	Liposomes cream	20	23.25	8.45	
1	1% Emblica liposomes cream	20	27.55	6.81	S*
	Liposomes cream	20	19.95	7.52	
2	1% Emblica liposomes cream	20	23.10	5.82	NS
	Liposomes cream	20	25.25	12.20	
4	1% Emblica liposomes cream	18	22.72	7.65	NS
	Liposomes cream	18	24.22	12.36	

NS Non significance

S* Significance at $p < 0.05$

Moisturizers play a significant role in the skin. They increase the skin elasticity and smooth the skin. While assessment of skin by clinical grading is still important to both moisturizer and mildness studies, currently there are many non invasive methods to evaluation of skin. The choice of method depends upon the characteristics of the skin to be quantified (Wickett *et al*., 2007). Several parameters such as, environment temperature, humidity and sun exposure, age, condition of the skin are associated with the water content of stratum corneum resulting in moisture of

the skin. A key concern in esthetic counseling is the determination of skin hydration in the corneal layer (Roberts and Wertz, 2008). Sufficient moisture makes the skin appear smooth, soft and supple. A lack of moisture can cause the skin to look dull and cracked, appearing older. Surface skin moisture measurement is performed by a capacitive sensor. The higher capacitance of the skin, the high scores and more hydrated skin is the aim of measurement.

Hydration of the stratum corneum can lead to profound changes in its barrier properties (Roberts and Walters, 1993; Walters and Roberts, 2007). There was no statistical significance in the skin moisture in the end of experiments (week 4). In week 2 of this study, the skin hydration was significant respectively. Not only resembling stratum corneum lipids composition of liposomes but also effects of vehicles in emulsions could have several benefits to improve skin hydration (Choi and Maibach, 2005; Patravale and Mandawgade, 2007; Roberts and Wertz, 2008; Otto *et al*., 2009). The compositions of emulsion act as emollient and occlusive properties, for this reason, it is therefore liposomes compositions and emulsion improve skin hydration for their mode of actions as well (Gabard, 2005). It is possible that the prominent hydration induced by liposomes and formulations immediately after application (week 2), initially improved the skin moist. However, the effects of moisture were only highlighted for a short time after application and soon decrease with time. This effect can be caused by the evaporation or the removal from the skin surface of formulations (Uhoda *et al*., 2008).

It can be described that chemicals and methods to reduce the barrier capability of the stratum corneum in order to promote skin penetration (Hadgraft, 1999). Hydration, water is the most widely used and safest method to increase skin

penetration of hydrophilic and lipophilic permeants (Forster *et al*., 2009). Additional water with the stratum corneum could alter permeant solubility and thereby modify partitioning from vehicle into membrane. Hydration can be increased by occlusion with plastic films; paraffins, oils, waxes as components of o/w emulsion that donate water (Benson, 2005). In this study, although it was not significant difference in week 4 results from hydration of liposomal effects. It was not surprisingly due to the methodological difficulties as possible (Miteva and Fluhr, 2008).

4.10.8 Elasticity

Table 19 significant differences of skin elasticity between 1% emblica liposomes cream and 1% liposomes cream

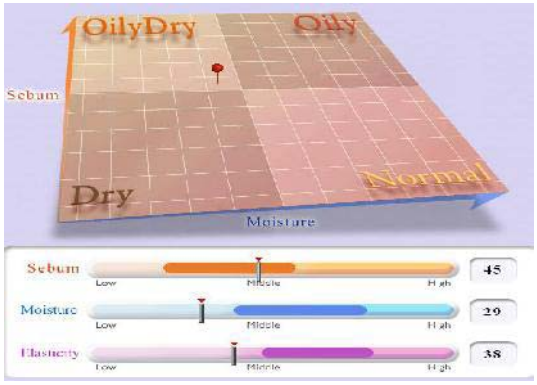
Week	Group	Subjects	Mean	SD	Sig
0	1% Emblica liposomes cream	20	46.45	9.48	NS
	Liposomes cream	20	43.85	9.35	
1	1% Emblica liposomes cream	20	52.25	8.24	S*
	Liposomes cream	20	41.90	9.66	
2	1% Emblica liposomes cream	20	49.70	8.78	S*
	Liposomes cream	20	41.75	10.08	
4	1% Emblica liposomes cream	19	50.27	8.94	S*
	Liposomes cream	19	43.22	8.34	

NS Non significance

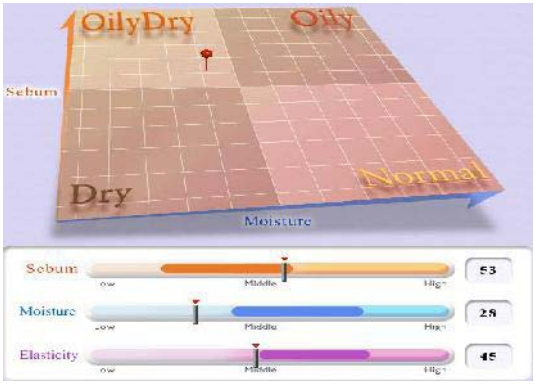
S* Significance at $p < 0.05$

Skin elasticity is determined by the elastin and collagen fibers of the dermis resulting from skin hydration, for this reason, it is associated with skin

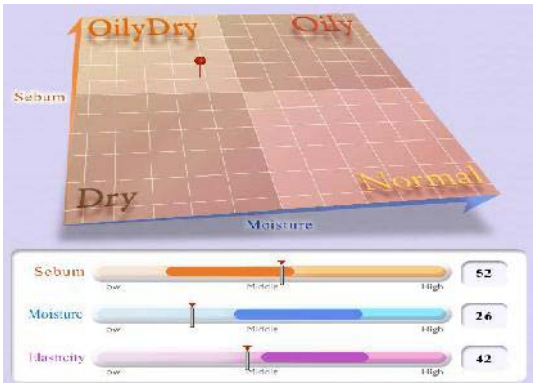
elasticity (Wiechers and Barlow, 1999). Because of ageing and external factors, the elasticity of the skin deteriorates over time, leaving the skin appearing dull and saggy. The elasticity sensor applies a unique measurement technique where a tiny sensor tip oscillates at a particular frequency and, when applied to the skin, will exhibit a chance in the frequency reflecting the firmness of the skin. Skin elasticity is determined by replacement i-Sensor on the facial.



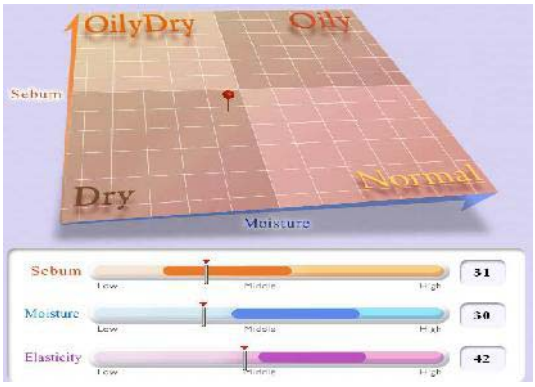
week 0



week 1



week 2



week 4

Figure 25 Skin sebum, skin moisture and skin elasticity

According to *in vivo* evaluation, it indicated that the application of 1% emblica liposomes cream could not be effective in skin structure, for instances; skin texture and skin orientation. Moreover, 1% emblica liposomes cream could not be

sufficient in order to use as an exfoliation cream and to increase skin elasticity due to keratin and elasticity value. Indicating a result 1% emblica liposomes cream could not improve not only skin moisture but also sebum as well. 1% emblica liposomes cream was obviously effective improvement, both skin dullness and degree of pigmentation, on the contrary.

A questionnaire was provided to volunteers and collected after one month. Moreover, volunteers were asked to give their recommendation on prompt parameters and their spontaneous comments were also recorded. The results were summarized in the followings;

Table 20 Evaluation of subjects compared with control

Parameters	1% emblica liposomes cream (n=20)	1% liposomes cream (n=20)	Significance
1. Color	4.45 ± 0.51	4.20 ± 0.69	0.262
2. Odor	4.40 ± 0.59	4.45 ± 0.68	0.649
3. Permeation	4.25 ± 0.63	4.15 ± 0.75	0.694
4. Viscosity	2.25 ± 1.20	3.00 ± 1.02	0.036*
5. Spreadability	2.55 ± 1.39	3.30 ± 1.12	0.114
6. Greasiness	2.05 ± 0.99	2.60 ± 0.94	0.045*
7. Sensation	4.60 ± 0.59	3.85 ± 0.67	0.000*
8. Irritation	4.30 ± 0.57	3.85 ± 0.87	0.965
9. Effectiveness	4.45 ± 0.51	3.70 ± 0.65	0.001*
10. Dosage form	4.40 ± 0.75	4.30 ± 0.57	0.649
11. Convenience	4.50 ± 0.15	4.10 ± 0.17	0.057
12. Prize	4.40 ± 0.15	4.10 ± 0.17	0.230
13. Satisfaction	4.50 ± 0.11	4.35 ± 0.15	0.419
14. Launching	4.60 ± 0.11	4.25 ± 0.16	0.031*

1 = no satisfaction, 2 = low satisfaction, 3 = moderate, 4 = satisfaction and 5 = very satisfaction

The results obtained from the answered questionnaires of volunteers that applied 1% emblica liposomes and 1% liposomes cream (control) after 1 month of twice daily use were concluded in the following:

Not only the color but also the odor for the average values both 1% emblica liposomes cream and 1% liposomes cream were not significant difference. Contrary to the viscosity obtained in this subjective evaluation in each group was not similar. Moreover, the permeation which is the basic concepts for cosmetic point of view was not different of active and control that blank liposomes was incorporated into formulations. The key conceptual in of cosmetic products was pointed out spreadability, greasiness and personal perception. There was not different of spreadability but statistically was different of greasiness and personal perception respectively. Safety evaluation of the products was evaluated before use at forearm targeted sites. The irritation was not found in this study in both groups, therefore; it can be concluded that 1% emblica liposomes cream and 1% liposomes cream were not irritated to skin. As previously shown for *in vivo* study found that 1% emblica liposomes cream was a good promising for cosmetic uses which related to the subjective assessments in effectiveness value 4.45 after application. For long term period of times, it is necessary to evaluate sensory evaluation in order to launch in the market. The results providing from volunteers were agree in the price and dosage forms of this product.

CHAPTER 5

CONCLUSION

In conclusion, emblica fruits extract is a natural antioxidant and rich of polyphenols. It also shows the nearly similar activity compared with a commercial antioxidant like ascorbic acid which is well known as various benefits in terms of EC_{50} = 2.48 ± 0.03 µg/mL. In this study, the model that used to estimate antioxidant activity was DPPH radical scavenging assay, according to EC_{50} emblica fruits extract was considerably as natural antioxidant sources to develop cosmetic products as well. The role of polyphenols from emblica fruits extract has a great deal of potential as part of a growing natural antiageing and whitening skin care market. With all natural ingredients, however, it is important to standardize extraction methods as well as assay for their activity. Chemical constituents in emblica fruits extract as usual which was believed containing largely amount of ascorbic acid and polyphenolic compounds. In this study, experimental conditions, separation of the ascorbic acid peak from interference of the matrix was unsatisfactory, therefore; to quantitative analysis by HPLC system; gallic acid was used as bioactive marker respectively.

In order to verify HPLC system analysis that modified from Kumaran and Karunakaran (2006) gallic acid was examined in validation assay. The HPLC method as previously described was a represented to reliable procedure for analysis of gallic acid in emblica fruits extract. Gallic acid was found to be a mainly compounds among phenolic acid in this study, this results agree with the previous reports,

however; the amount of gallic acid in ethanolic emblica fruits extract was not nearly as reported that used ethyl acetate performed by Kumaran and Karunakaran (2006). HPLC technique is a precise, specific and accurate for the determination of gallic acid.

According to the study of Nobborb (2008) who reported the antioxidant capacity of emblica fruits extract were not significant changes in phenolic contents of the extract at pH 5.5 and the activity was higher in acidic solution. From this point of view, the formulations like creams was necessary to formulate the nearest pH 5.5 in order to maintained antioxidant activity and suitable for skin. According to the structure of emblica fruits extract and the issue of properties, it might be irritated to skin and limitation of permeability that were main problems importantly in cosmetic. The novel cosmetic delivery system as liposomes was selected to combat this point of views. Emblica fruits extract liposomes were successfully prepared by use the master formula from Nobnorb (2008), in short; comprising SPC: Tween 80: DA (84: 16: 2.5; weight ratio) with total lipid of 200 µmol/mL concentration of emblica extract was 1% by weight. Emblica liposomes are brown in terms of color and looked like viscousness. Moreover, as shown from *in vitro* study, it should be noted that when administrated through novel delivery system show much better skin permeation profile.

Despite the fact that the hydration effect resulting from liposomes contributes to the healthy appearance of the skin, it also influences directly the percutaneous absorption. For cosmetic applications it is important that the active causing adverse effects are not systemically absorbed; however, it is crucial a certain permeation into the skin for the desired effects.

Moreover, the clinical study clearly demonstrated that the emblica liposomes cream was able to use as skin whitening products in human volunteers. Emblica fruit extract apparently showed the efficiency onset of significant whitening effect that required only 2-4 weeks of application. After the four weeks of treatment, reduction in skin pigmentation was seen in 15 out of 20 subjects (80%) which was statistically significant ($p<0.05$). To evaluate skin whitening in terms of skin dullness and degree of pigmentation showed statistically significant as well. Considering the low concentrations employed in this study (1% emblica liposomes cream), the extract may have a very promising potential for use as a safe, effective and economical whitening agent. It is interestingly to note that there was not different in moisture content in the subjects. Moreover, satisfaction of subjects was collected and found that the subjects satisfied in 1% emblica liposomes cream in effectiveness and sensation as well. Finally, topical application of cosmetic is very promising as it allows controlled and continuous delivery into the skin.

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