การพัฒนาสูตรตำรับนิโอโซมที่บรรจุสารสกัดมะขามป้อมซึ่งมีฤทธิ์ทำให้ผิวขาว
และต้านอนุมูลอิสระ

นางสาวเสาวลักษณ์ เลิศอมรเสถียร

FORMULATION OF NIOSOMES CONTAINING *PHYLLANTHUS EMBLICA* EXTRACT WITH WHITENING AND FREE RADICAL SCAVENGING ACTIVITIES

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                    WHITENING AND FREE RADICAL SCAVENGING
                    ACTIVITIES
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เสาวลักษณ์ เลิศอมรเสถียร: การพัฒนาสูตรตำรับนิโอโซมที่บรรจุสารสกัดมะขามป้อมซึ่งมีฤทธิ์ทำให้ผิวขาวและต้านอนุมูลอิสระ. (FORMULATION OF NIOSOMES CONTAINING *PHYLLANTHUS EMBLICA* EXTRACT WITH WHITENING AND FREE RADICAL SCAVENGING ACTIVITES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ภญ.ดร.สุชาดา ชุติมาวรพันธ์, 200 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระและทำให้ผิวขาวของสารสกัดมะขามป้อม พัฒนาสูตรตำรับและประเมินลักษณะของนิโอโซมที่บรรจุสารสกัดมะขามป้อมซึ่งเตรียมโดยวิธีระเหยแบบกลับวัตภาค ได้ศึกษาผลของชนิดสารลดแรงตึงผิว และอัตราส่วนของสารลดแรงตึงผิว:คอเลสเตอรอล ต่อการเตรียมและคุณสมบัติทางเคมีกายภาพของนิโอโซม สารสกัดมะขามป้อมเตรียมได้ในรูปผงจากการทำแห้งเยือกแข็ง ฤทธิ์ต้านอนุมูลอิสระโดยวิธีดีพีพีเอชของสารสกัดมะขามป้อมซึ่งมีสารสำคัญคือกรดแกลลิค มีค่าความเข้มข้นที่ฤทธิ์ 50% เท่ากับ 1.38±0.01 ไมโครกรัมต่อมิลลิลิตร ซึ่งมีฤทธิ์สูงกว่ากรดแอสคอร์บิค โดยกรดแกลลิคมาตรฐานมีฤทธิ์สูงสุด ส่วนฤทธิ์ต้านไทโรซิเนสนั้น สารสกัดมะขามป้อมมีฤทธิ์ต่ำกว่ากรดโคจิค แต่มีฤทธิ์สูงกว่าแอลฟาแอลบูทิน โดยมีค่าความเข้มข้นที่ฤทธิ์ 50% เท่ากับ 0.97±0.06 มิลลิกรัมต่อมิลลิลิตร การเตรียมนิโอโซมโดยบรรจุสารสกัดมะขามป้อม 0.5% โดยน้ำหนักต่อปริมาตร โดยวิธีระเหยแบบกลับวัตภาค สามารถเตรียมได้จำนวน 9 สูตรตำรับโดยใช้ สแปน 20 สแปน 40 สแปน 60 และสแปน 80 โดยเปลี่ยนอัตราส่วนของสารลดแรงตึงผิว:คอเลสเตอรอล เท่ากับ 1:1, 6:4, 7:3 และใช้โซลูแลนซี-24 เป็นสารเพิ่มความคงตัว ขนาดเวสิเคิลที่ได้จากทุกสูตรตำรับซึ่งวัดโดยเทคนิคโฟตอนคอร์รีเลชัน สเปกโทรสโกปี มีขนาดระหว่าง 215-306 นาโนเมตร ขนาดนิโอโซมที่ได้จากสูตรตำรับที่ใช้ สแปน 20 (ค่าเอชแอลบี 8.6) ซึ่งมีสายโซ่แอลคิลสั้นที่สุด (12 คาร์บอน) มีขนาดใหญ่กว่าที่เตรียมได้จากสแปน 40 (ค่าเอชแอลบี 6.7, 16 คาร์บอน) สแปน 60 (ค่าเอชแอลบี 4.7, 18 คาร์บอน) สูตรตำรับที่ประกอบด้วย สแปน 20: คอเลสเตอรอล: โซลูแลนซี-24 เท่ากับ 57:38 :5 โดยโมล มีค่าประสิทธิภาพการกักเก็บกรดแกลลิคสูงที่สุด เท่ากับ 49.29±3.73 % มีขนาดอนุภาคเฉลี่ยเท่ากับ 273±7.9 นาโนเมตร และดัชนีพอลิดิสเพิสซิตี เท่ากับ 0.333±0.061 ขนาดเวสิเคิลมีการเปลี่ยนแปลงใหญ่ขึ้นเล็กน้อยเมื่อเก็บไว้ที่อุณหภูมิ 4°ซ และ30°ซ นาน 12 สัปดาห์ ยกเว้นสูตรตำรับที่ใช้สแปน 80 พบว่าเกิดการแยกวัตภาคเมื่อเก็บไว้ ค่าเปอร์เซนต์ปริมาณกรดแกลลิคในสูตรตำรับนิโอโซมที่เก็บที่อุณหภูมิ 4°ซ มีค่าลดลงเนื่องจากการเสื่อมสลายของกรดแกลลิค น้อยกว่าที่ 30°ซ การพัฒนาสูตรตำรับซีรัมซึ่งผสมนิโอโซมที่บรรจุสารสกัดมะขามป้อมเตรียมได้โดยใช้พอล็อกซาเมอร์ 407 เป็นสารเพิ่มความหนืด ในปริมาณ 16 % โดยน้ำหนักต่อปริมาตร การศึกษาการซึมผ่านผิวหนังแบบนอกกายใช้ผิวหนังหน้าท้องของสุกรแรกเกิดเป็นผิวหนังโมเดล พบว่านิโอโซมสามารถเพิ่มการซึมผ่านผิวหนังได้สูงกว่าสูตรตำรับเดียวกันในรูปซีรัม พบว่า นิโอโซมที่ประกอบด้วย สแปน 20: คอเลสเตอรอล: โซลูแลนซี-24 เท่ากับ 57:38 :5 โดยโมล เพิ่มการซึมผ่านผิวหนัง โดยมีค่าสัมประสิทธิ์การซึมผ่าน เท่ากับ 0.0025±0.0009 ซม/ ชั่วโมงและมีปริมาณเปอร์เซนต์กรดแกลลิคที่สะสมในผิวหนัง เท่ากับ 2.97±0.59 %

ภาควิชา <u>วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม</u> ลายมือชื่อนิสิต.......................................

สาขาวิชา <u>เภสัชกรรม</u>..ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก......

ปีการศึกษา <u>2553</u>...

5176603533 : MAJOR PHARMACEUTICS

KEYWORDS: *PHYLLANTHUS EMBLICA* / NIOSOMES / ANTI-FREE RADICAL ACTIVITY / ANTI-TYROSINASE ACTIVITY / SKIN PERMEATION

SAOWALAK LEARTAMONSTIEAN: FORMULATION OF NIOSOMES CONTAINING *PHYLLANTHUS EMBLICA* EXTRACT WITH WHITENING AND FREE RADICAL SCAVENGING ACTIVITIES. ADVISOR: ASSOC. PROF. SUCHADA CHUTIMAWORAPAN, Ph.D., 200 pp.

The purposes of this study were to investigate anti-free radical and whitening activities of *Phyllanthus emblica* extract, to formulate and characterize niosomes containing emblica extract prepared by reverse phase evaporation method. Effect of surfactant type and surfactant : cholesterol ratio on the preparation and physicochemical properties of niosomes was studied. The emblica extract was prepared as lyophilized powder. The DPPH scavenging activity of emblica extract, which contained the main constituent namely gallic acid, with the IC_{50} of $1.38\pm0.01\mu g/ml$, was higher than ascorbic acid, whereas the standard gallic acid had the greatest activity. Embica extract exhibited lower antityrosinase activity than kojic acid, but much higher than alpha arbutin, with IC_{50} of 0.97 ± 0.06 mg/ml. Consequently, niosomes loaded with 0.5% weight by volume emblica extract could be prepared by reverse phase evaporation technique. Niosomes could be achieved by nine formulations using Span 20, Span 40, Span 60 and Span 80 with varied surfactant: cholesterol ratios of 1:1, 6:4, 7:3 and with Solulan C-24 as stabilizer. Vesicular size of all formulations determined by photon correlation spectroscopy technique were between 215- 306 nanometers. The size obtained from Span 20 (HLB 8.6) with shortest alkyl chain (C12), was larger than from Span 40 (HLB 6.7, C16) and Span 60 (HLB 4.7, C18). The formulation comprised of Span 20: cholesterol : Solulan C-24 of 57:38:5 by mole, showed the highest entrapment efficiency of gallic acid as 49.29 ± 4.60 %, mean size of 273 ± 7.9 nanometers and polydispersity index of 0.333 ± 0.061. Vesicular size showed a little larger mean sizes at both 4°C and 30°C at 12 weeks, except the formulation with Span 80 showed phase separation after storage. The percentage content of gallic acid in niosomes decreased due to the degradation of gallic acid in a less amount at 4°C than 30°C . The serum formulation with loaded emblica extract niosomes was prepared with 16 % weight by volume of poloxamer 407 as viscosity enhancer. The in vitro permeation using newborn abdominal porcine skin as skin model revealed that niosomes showed higher permeation than its corresponding serum formulation. The niosomes comprised of Span 20: cholesterol : Solulan C-24 of 57:38:5 by mole enhanced the skin permeation as shown by the highest permeability coefficient of 0.0025 ± 0.0009 cm/h, and highest percentage gallic acid deposited in the skin as $2.97\pm0.59\%$.

Department : <u>Pharmaceutics and Industrial Pharmacy</u>	Student's Signature ....

Field of Study : <u>Pharmaceutics</u>	Advisor's Signature ....

Academic Year : <u>2010</u> ....

ACKNOWLEDGEMENTS

CONTENTS

CHAPTER	**Page**

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	=	analysis of variance
Abs	=	absorbance
AUC	=	area under curve
°C	=	degree celcius
C_d	=	concentration of drug in donor
cm	=	centimeter
cps	=	centipoise
C.V.	=	coefficient of variation
DPPH	=	1,1-diphenyl-2-picrylhydrazyine
et al.	=	*et alii*, and others
g	=	gram
h	=	hour
h	=	thickness of membrane
HPLC	=	high performance liquid chromatography
K	=	partition coefficient
kg	=	kilogram
LUV	=	large unilamellar vesicles
mbar	=	millibar
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
MW	=	molecular weight
n	=	sample size
nm	=	nanometer
P	=	permeability coefficient
PBS	=	phosphate buffer saline
pH	=	the negative logarithm of the hydrogen ion
R^2	=	coefficient of determination

ROS	=	reactive oxygen species
REVs	=	reverse phase evaporation vesicles
rpm	=	revolution per minute
RT	=	room temperature
SD	=	standard deviation
Sec	=	second
SPSS	=	statistical package for the social science
t	=	time
Tc	=	transition temperature
TEM	=	transmission electron microscopy
TLC	=	thin layer chromatography
μg	=	microgram
μg/ml	=	microgram per mililitre
μl	=	microlitre
μm	=	micrometer
μmol	=	micromole
UV	=	ultraviolet
w/v	=	weight by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Nowsaday, the use of whitening agents in cosmeceutical products are interested in Asia. The most popular whitening agents such as alpha-hydroxy acid, arbutin and kojic acid are used worldwide. The higher manufacturing cost and toxicity of synthetic whitening agents have created a need for identifying alternative natural substances. Natural substances are presumed to be safe since they occur in plants such as mulberry extract, licorice extract and amla extract (Zhu and Gao, 2008).

Phyllanthus emblica L. or Indian gooseberry or "Ma-khaam Pom" in Thai or "Amla" in Hindi is widely distributed in subtropical and tropical areas of China, India , Indonesia, Malaysia and Thailand (Liu et al., 2008). The fruits of *Phyllanthus emblica* are used in the Aryuveda and believed to increase defense against diseases. It was reported to have hypolipidemic (Yokozawa et al., 2007) and hypoglycemic activity (Khan, 2009). In addition, a major compound of *Phyllanthus emblica* as gallic acid (Kumaran and Karunakaran, 2006) was reported to be antimicrobial (Mayachiew and Devahastin, 2008), hepatoprotective in Wistar rats (Khan, 2009) and wound healing (Sumitra et al., 2009). Inclusion, many researchers reported emblica extract was antioxidant activity (Kumaran and Karunakaran, 2006; Mayachiew and Devahastin, 2007; Liu et al., 2008; Luo et al., 2009). Moreover, emblica extract was shown to have antityrosinase and anticollagenase activities (Jithavech, 2005). Furthermore, gallic acid has a strong inhibitory activity to tyrosinase which is a specific enzyme for melanogenesis (Kim, 2007). The enzyme is located in the melanocytes of the epidermis (Szabo, 1957) that involved to eumelanogenesis. The enzymatic oxidation of tyrosine to it corresponding o-dopaquinone catalyzed by tyrosinase which leads to the synthesis of eumelanins (Kim and Uyama, 2005). In cell culture, gallic acid was indicated that non-cytotoxicity in B16 melanoma cells and inhibitory potency on mushroom tyrosinase was significantly higher compared to kojic acid (Kim, 2007).

Stratum corneum is a main barrier of many compounds passing through the skin. Several approaches have been developed to weaken this skin barrier. One

possibility for increasing the penetration of drugs and many cosmetic ingredients is the use of vesicular system, such as liposomes and niosomes (Manosroi, Jantrawut and Manosroi, 2008).

Niosomes are non-ionic surfactant based vesicles that have been developed as alternative controlled drug delivery system to liposomes in order to overcome the problems associated with large-scale production, cost-effectiveness and stability. They consist of hydrated mixtures of cholesterol, charge inducing substance, and non-ionic surfactants (Sahin, 2007). These penetration enhancers are biodegradable, non-toxic, amphiphilic in nature (Choi and Maibach, 2005). They can entrap hydrophilic drug and other bioactives upon encapsulation or hydrophobic material by partitioning of these molecules into hydrophobic domains. There are many substances which used this system such as acyclocir (Attia et al., 2007), ofloxacin (Hosny, 2009), genetic immunization against hepatitis B (Vyas et al., 2005) and diclofenac sodium (Naresh et al., 1994). Since this system has low viscosity, the addition of gelling agent to enhance viscosity was aimed to control and direct delivery of the drug to the site of action. To date, there is no report of the investigation of preparation of niosomes and niosomal serum loaded with *Phyllanthus emblica* extract and its physicochemical properties. Therefore, we need to investigate formulation factors which affect to the preparation and properties of niosomes and niosome serums.

The main purposes of this present study were to attempt to prepare *Phyllanthus emblica* extract in lyophilized powder form and evaluated its whitening and antioxidant activities. Afterthat, the preparation and characterization of niosomes and niosome serums containing *Phyllanthus emblica* extract were investigated.

The purposes of this study were as follows :

1. To evaluate in vitro whitening and antioxidant activities of *Phyllanthus emblica* extracts.

2. To study the effect of type and quantity of surfactants on physicochemical properties and stability of *Phyllanthus emblica* extract loaded in niosomes.

3. To investigate and compare in vitro skin permeation of *Phyllanthus emblica* extract loaded in niosomes and serums.

CHAPTER II

LITERATURE REVIEW

A. Botanical, Chemical and Pharmacological Aspects of *Phyllanthus emblica* L.

1. Botanical aspects of *Phyllanthus emblica*

Phyllanthus emblica L. (synonym: *Emblica officinalis* Gaertn.) belongs to the family Euphorbiaceae. It bears such local vernacular names as emblic, amla, Malacca tree and Indian gooseberry. The Thai name for the fruit is Ma-khaam Pom.

The emblic tree, native in subtropical and tropical southeastern asia, particularly in China, central and southern India, Pakistan, Malaysia, Indonesia and Thailand (Liu et al., 2008). The emblic tree reaches a height of 60 feet and, in rare instances, 100 feet. While actually deciduous, sheding its branchlets as well as its leaves, it is seldom entirely bare and is therefore often cited as an evergreen. Its fairly smooth bark is a pale grayish-brown and peels off in thin flakes like that of the guava. The miniature, oblong leaves, only 1/8 inch wide and 1/2 to 3/4 inch long, distichously disposed on very slender branchlets, give a misleading impression of finely pinnate foliage. Small, inconspicuous, greenish-yellow flowers are borne in compact clusters in the axis of the lower leaves. Usually, male flowers occur at the lower end of a growing branchlet, with the female flowers above them.

The nearly stemless fruit is round, indented at the base, and smooth, though six pale lines, sometimes faintly evident as ridges, extending from the base to the apex, gives it the appearance of being divided into six segment or lobes. Light green at first, the fruit becomes a dull greenish-yellow or, more rarely, brick-red as it matures (Morton, 1960). The fruits of Ma-khaam Pom is shown in Figure 1 .

Figure 1. *Phyllanthus emblica* Linn. (Family Euphorbiaceae) (Jaijoy et al., 2010)

2. Chemical components of *Phyllanthus emblica* L.

The *P.emblica* L. tree contains the different classes of constituents in Table 1. The complexity of the mixture of compounds can make the isolation and identification of the substances present in this genus very laborious. The choice of solvent in the isolation of compounds is corresponded to the polarity of compounds that are required.

Table 1. The classes of chemical constituents reported in *Phyllanthus emblica* (Summanen, 1999)

Class	Compound	Part of plant
Alkaloid	Phyllantine	fruits, leaves
	Phyllantidine	fruits, leaves
	Zeatin	leaves
	Zeatin nucleotide	fruits
	Zeatin riboside	fruits
Benzenoid	Chebulic acid	leaves
	Chebulinic acid	leaves

Table 1. The classes of chemical constituents reported in *Phyllanthus emblica* (Summanen, 1999) (continued)

Class	Compound	Part of plant
Benzenoid	Chebulagic acid	leaves
	Gallic acid	fruits, leaves
	Ellagic acid	fruits, leaves
	Amalaic acid	fruits
	Corilagin	fruits
	3-6-di-O-galloyl-glucose ethyl gallate	fruits
	B-glucogallin	fruits, leaves
	1,6-di-O-galloyl-β-D-glucose	fruits
	Putranjivain A	fruits
	Digallic acid	fruits
	Phyllemblic acid	fruits
	Emblicol	fruits
	Music acid	fruits
Furanolactone	Ascorbic acid	fruits
Diterpene	Gibberellins A-1	leaves
	Gibberellins A-3	leaves
	Gibberellins A-4	leaves
	Gibberellins A-7	leaves
	Gibberellins A-9	leaves
Triterpene	Lupeol	fruits, leaves
Flavonoid	leucodelphinidin	leaves
	Kaempherol	leaves
	Kaempherol-3-glucoside	leaves
	Rutin	leaves
	Quercetin	leaves
	Kaempherol-3-O-β-D-glucoside	fruits
	Quercetin-3-O-β-D-glucoside	fruits
Sterol	β-sitosterol	leaves

Table 1. The classes of chemical constituents reported in *Phyllanthus emblica* (Summanen, 1999) (continued)

Class	Compound	Part of plant
Carbohydrate	Acidic and neutral polysaccharides	fruits
	Glucose	leaves

The composition in fruit of *P.emblica* are reported in Table 2 (Kumaran and Karunakaran, 2006).

Table 2. The composition in fruits of *Phyllanthus emblica*.

Compounds	Amount[a]
Gallic acid	71.89 %
Methyl gallate	34.82 %
Corilagin	32.48 %
Geraniin	21.12 %
Furosin	7.91 %

[a] The percentage amount compared with dry weight of fruits

3. **Physicochemical properties of gallic acid** (National library of medicine HSDB database)

Synonym

3,4,5-Trihydroxybenzoic acid ($C_7H_6O_5$)

Structure of gallic acid

Gallic acid is a chemical based on the structure of phenol which is a molecule with a ring structure containing 6 carbons with a –OH (hydroxyl group) attached to one of the carbons.

Gallic acid has 3-OH groups and one –COOH (carboxylic group) attached to the ring. The chemical structure of gallic acid is depicted in Figure 2.
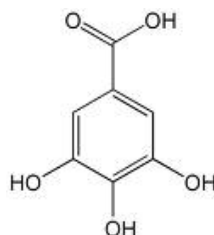
Figure 2. Chemical structure of gallic acid (Kumaran and Karunakaran, 2006)

Description.

Gallic acid is colorless or slightly yellow crystalline needles or prisms.

Molecular weight : 170.12

Dissociation constants : pKa = 4.40

Octanol/Water Partition Coefficient : Log P = 0.7

Solubility

Gallic acid is soluble in ethyl acetate,1 g with water 87 ml, 3 ml boiling water, 6 ml alcohol, 100 ml ether, 10 ml glycerol, 5 ml acetone, practically insoluble in benzene, chloroform, petroleum ether.

4.   Pharmacological activities of *Phyllanthus emblica* L.

The fruits of *P.emblica* are consumed as fruit or in the form of food products. The fruit is widely used in the Ayurveda and believed to increase defense against disease.

4.1. Hepatoprotective and gastroprotective activity

The fruits of *P.emblica* have been reported to be used for hepatoprotection in Ayurveda. *Phyllanthus emblica* extract was investigated on ethanol induced hepatic injury in rats. It demonstrated hepatoprotective action (Pramyothin et al., 2006). Hepatoprotective effect of 50% hydroalcoholic extract of the fruits of *P.emblica* against antituberculosis (anti-TB) drugs-induced liver toxicity has been reported. The extract exhibits hepatoprotective activity due to its membrane stabilizing, antioxidative activity and CYP 2E1 inhibitory effect (Tasduq et al., 2005).

The ethanolic extract of *P.emblica* fruits was examined for its antisecretory and antiulcer activities in rats, including pylorus ligation, indomethacin, hypothermic restraint, stress-induced gastric ulcer and ulcers from necrotizing agents (80% ethanol, 0.2 M NaOH and 25% NaCl). The extract decreased the pyloric-ligation induced basal gastric secretion, titratable acidity and gastric mucosal injury that developed to gastric lesions. Moreover, the extract offered protection against ethanol induced depletion of stomach wall mucus and reduction in nonprotein sulfhydryl concentration. It was

reported that the extract exhibited antisecretory, cytoprotective and antiulcer properties (Al-Rehaily et al., 2002).

4.2. Antimicrobial activity

The extract of *P.emblica* fruits have been reported its antibacterial activity. The aqueous infusion and decoction of *P.emblica* fruits exhibited potent antimicrobial activity against *Staphylococcus haemolyticus, Staphylococcus saprophyticus, Micrococccus varians, Micrococccus lylae, Micrococccus roseus, Micrococccus halobius, Micrococccus sedenterius, Bacillus subtilis* and *Bacillus megaterium*. The aqueous infusion of *P.emblica* fruits exhibited maximum activity against *Bacillus subtilis* with 20.46 mm mean zone of inhibition and aqueous decoction exhibited maximum activity against *Staphylococcus haemolyticus* with 23.32 mm mean zone of inhibition. The minimum activities of both aqueous infusion and decoction of *P.emblica* were found against *Candida albicans* with 10.56 mm and 12.32 mm, respectively (Saeed and Tariq, 2007). In addition, the maximum inhibition zone, minimum inhibitory concentration and minimum bactericidal concentration values for *Staphylococcus aureus* were 21.8 mm, 13.97 mg/ml and 13.97 mg/ml of ethanol extract of *P.emblica* fruits, respectively (Mayachiew and Devahastin, 2007).

4.3. Antidiabetic activity

Oral administration of the extract (100 mg/kg body weight) reduced the blood sugar level in normal and in alloxan (120 mg/kg) diabetic rats significantly within 4 hours. *P.emblica* and an enriched fraction of its tannoids were effective in delaying development of diabetic cataract in rats (Suryanarayana et al., 2007). *P.emblica* was proved as an important inhibitor of aldose reductase which involved in the development of secondary complications of diabetes, sugar cataracts (Suryanarayana et al., 2004).

4.4. Antidiarrhea activity

In Indian medicine, the dried fruits are used to treat diarrhea, and dysentery. A sherbet made of the fruits was reported to arrest acute bacillary dysentery. The methanol extract of *P.emblica* fruits was evaluated in Wistar albino rats. The extract

exhibited dose-related antidiarrheal activity at doses of 50, 100, and 150 mg/kg on diarrhea induced by castor oil or magnesium sulfate. The extract had a similar activity as loperamide when tested at doses of 50, 100, and 150 mg/kg and significantly inhibited the frequency of defecation and the wetness of the fecal droppings when compared to control rats. The extract produced a significant reduction in gastrointestinal motility in charcoal meal tests in rats. It also significantly inhibited PGE2-induced enteropooling as compared to control animals (Perianayagam et al., 2005). The inhibitory effect of the extract justifies the use of the plant as a nonspecific antidiarrheal agent in folk medicine.

4.5. Antioxidant activity

Free radicals play an important role in phatogenesis of some serious diseases, such as neurodegenerative disorders, cancer, liver cirrhosis, cardiovascular disease, artherosclerosis, cataracts, diabetes and inflammation.

The ethylacetate extract of *P.emblica* dried fruits exhibited strong 1,1'-diphenyl-2-2'-picrylhydrazyl (DPPH) radical scavenging activities. The IC_{50} values of ethylacetate extract, vitamin C and butylated hydroxytoluene (BHT) were 8.05, 17.17 and 39.29 μg/ml, respectively. The potency of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) scavenging activity were expressed as Trolox® equivalent antioxidant capacity (TEAC). The TEAC values of ethylacetate extract, BHT, 6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid (Trolox®) and vitamin C were 1.56, 1.23, 1 and 0.92 μg/ml. The DPPH radical and ABTS scavenging activities of ethylacetate extract were higher than vitamin C, BHT and Trolox® (Luo et al., 2009). In nitric oxide scavenging activity, ethylaceate extract showed strong nitric oxide scavenging activity than hexane and water extract. In the ethylacetate extract, gallic acid was found to be a major compound that showed the highest NO scavenging activity (Kumaran and Karunakaran, 2006).

The aqueous extract showed ability to inhibit γ-radiation-induced lipid peroxidation (LPO) in rat liver microsomes and superoxide dismutase (SOD) damage in rat liver mitochondria. The aqueous extract acts as a very good antioxidant against γ-radiation induced LPO which measured in terms of thiobarbituric acid reactive

substances. Similarly, it was found to inhibit the damage to antioxidant enzyme SOD (Khopde et al., 2001).

4.6. Reducing cholesterol and dyslipidemia

The fresh juice of *P.emblica* fruits was evaluated in cholesterol-fed rabbits (rendered hyperlipidaemic by atherogenic diet and cholesterol feeding) to lipid lowering and antiatherosclerotic. The juice at a dose of 5 ml/kg body weight per day for 60 days was found to decrease serum cholesterol, triglyceride, phospholipid and low-density lipoprotein (LDL) levels by 82%, 66%, 77% and 90%, respectively. *P.emblica* juice is an effective hypolipidaemic agent and can be used as a pharmaceutical tool in hyperlipidaemic subjects (Mathur et al., 1996). Besides, the *P.emblica* extract reduced LDL oxidation and cholesterol levels in $Cu^{(2+)}$-induced LDL oxidation and cholesterol-fed rats (Kim et al., 2005).

4.7. Antipyretic and analgesic activities

The ethanol and aqueous extracts of dried fruits of *P.emblica* were investigated for anti-pyretic and analgesic activity. Both extracts at a dose of 500 mg/kg intraperitoneally showed significant reduction in brewer's yeast induced hyperthermia in rats similar to aspirin. The extracts elicited pronounced inhibitory effect on acetic acid-induced writhing response in mice in the analgesic test. This might be due to the presence of tannins, alkaloids, phenolic compounds, amino acids and carbohydrates (Perianayagam et al., 2004).

B. Antioxidant Activity

Free radicals are types of reactive oxygen species (ROS), which include all highly reactive, oxygen-containing molecules. Types of ROS include the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All these radicals are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage. Free radicals may be defined as chemical species associated with an odd or unpaired electron. They are neutral, short lived, unstable and highly reactive to pair up the odd electron and finally achieve stable configuration. They are capable of attacking the healthy cells of the body, causing them to lose their structure and function. Cell damage caused by free radicals appears to be a major contributor to aging and degenerative diseases of aging (Lee, Koo and Min, 2004). Many methods are available to determine antioxidant activity in plants, it is important to employ a consistent and rapid method. It has been found that the most common and reliable method are the ABTS and DPPH methods (Krishnaiah, Sarbatly and Nithyanandam, 2010).

1.   1,1-Diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolourizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 517 nm. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound (Luo et al., 2009).

2.   2,2′-Azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging assay

The ABTS radical scavenging method is one of the most extensively used antioxidant assays for plant samples. The ABTS radical cation is generated by the oxidation of ABTS with potassium persulfate, and its reduction in the presence of

hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm. This decolourisation assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances. The effect of the antioxidant concentration and the duration of the inhibition of the radical cation's absorption are taken into account when the antioxidant activity is determined. The activity is expressed in terms of the Trolox-equivalent antioxidant capacity of the extract (TEAC/mg) (Krishnaiah et al., 2010).

3.   Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay uses an oxidizable protein substrate and a peroxyl radical generator or a $Cu^{2+}-H_2O_2$ system as a hydroxyl radical generator. To date, it is the only method that takes the free radical reaction to completion and uses an area under the curve (AUC) technique for quantification, thereby combining both the inhibition percentage and the length of the inhibition time of the free radical's action into a single quantity. The assay has been widely used in many recent studies of plants (Krishnaiah et al., 2010).

C. Anti-tyrosinase Activity

1. Melanin formation

Melanin is one of the most widely distributed pigment and is found in bacteria, fungi, plants and animals. The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis (Szabo, 1957). The melanin synthesized by the melanocyte and distribution in the surrounding keratinocytes determines the actual color of the skin. Melanin is formed through a series of oxidative reactions involving the amino acid tyrosinase in the presence of tyrosinase (Kim and Uyama, 2005).

The biosynthesis pathway of melanin formation in various life forms is shown in Figure 3.
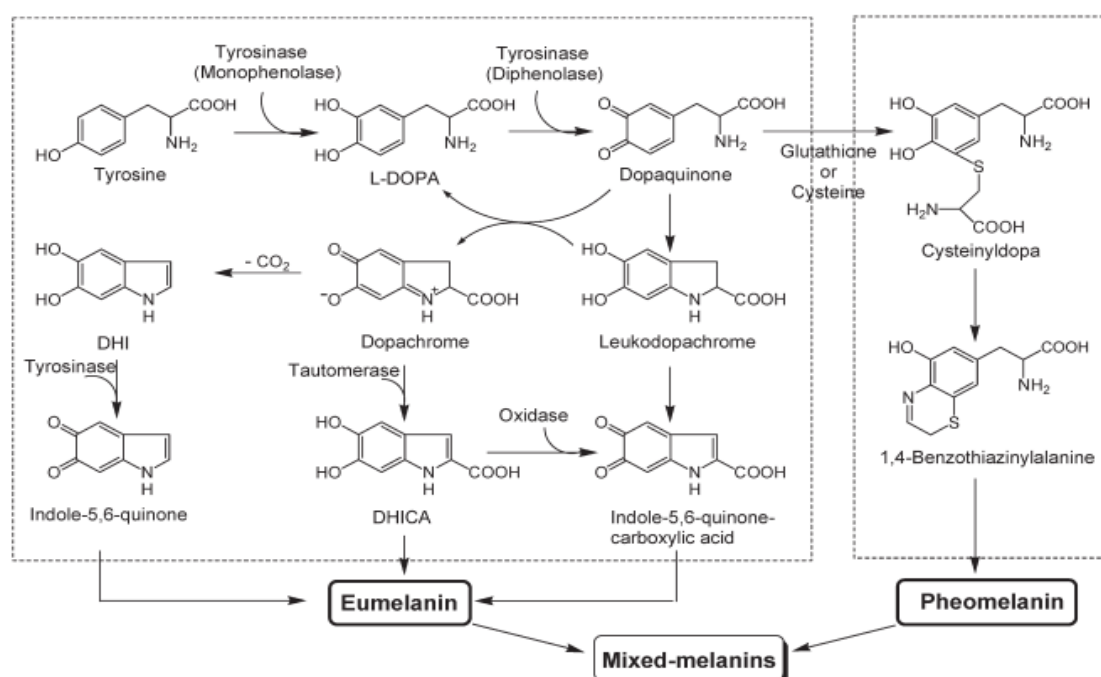
Figure 3. Biosynthetic pathway of melanin (Kim and Uyama, 2005)

Synthesis of melanin starts from the conversion of the amino acid L-tyrosine (monophenols) to 3,4-dihydroxyphenylalanine (o-phenols, L-DOPA) by tyrosinase enzyme with hydroxylation reaction and then oxidation of L-DOPA yields dopaquinone (o-quinones) by tyrosinase. These quinones are highly reactive and tend to polymerize spontaneously to form brown pigments of melanin. Quinones can also

react with amino acid and proteins and thus enhance the development of brown color (Nerya et al., 2004). Two types of melanin are eu-melanins (dark-brown) and pheomelanins (yellow-brown) which leading to the formation of eu-melanins is interesting. According to melanogenesis pathway, tyrosinase activity is thought to be a major regulatory factor in the initial steps of this pathway (Kim and Uyama, 2005).

2. Tyrosinase inhibitor (Kim and Uyama, 2005; Chang, 2009)

A number of tyrosinase inhibitors from both natural and synthetic sources have been identified.

2.1. Kojic acid

Kojic acid is a fungal metabolite that current used as a cosmetic skin whitening agent and as a food additive for preventing enzymatic browning. Kojic acid is the most intensively studied inhibitor of tyrosinase (Chang, 2009). Kojic acid is able to reduce dopaquinone to L-DOPA to prevent the final pigment forming and be oxidized to a yellow product by chemical interaction with dopaquinone (Kim and Uyama, 2005). Kojic acid effectively inhibited the formation of pigmented products.

2.2. Polyphenols

Polyphenols represent a diverse group of compounds containing multiple phenolic functionalities and are widely distributed in nature. Polyphenols are also the largest groups in tyrosinase inhibitors until now. Flavonoids are among the most numerous and best studied polyphenols including *Morus alba*, *Atrocarpus heterophyllus*, *Polygonum hydropiper* (Chang, 2009), *Broussonetia papyrifera* (Zheng et al., 2008).

2.3. Benzaldehyde and benzoate derivatives

In the past decade, a large number of benzaldehyde and benzoate derivatives have been isolated from plants and identified as tyrosinase inhibitor, including anisic acid, cinnamic acid, p-coumaric acid from the leaves of *Panax ginseng* and vanillic acid and it derivatives from black rice bran.

Gallic acid (3,4,5-trihydroxybenzoate) has been isolated and identified as a tyrosinase inhibitor from many plants. Gallic acid inhibited diphenolase activity

of mushroom tyrosinase with a IC_{50} value of 4500 µM, which is 100-fold lower than that of kojic acid (Chang, 2009).

2.4. Other natural and synthetic inhibitors

Recently, anthraquinones from different plant sources have been widely used since ancient times due to their laxative and cathartic properties. An anthraquinone was found to show similar tyrosinase inhibitory activity with that of kojic acid. In addition to the inhibitors from plants, marine beings inhibit virtually any environment in the sea, and they have been shown to produce novel substances with utilities in fine chemicals, drugs, and cosmetic products, including tyrosinase inhibitors. One phloroglucinol derivative, dieckol, was isolated from a marine brown algae, *Ecklonia stolonifera*, and displayed three times more activity than that of kojic acid.

D. Niosomes

Niosomes are non-ionic surfactant based vesicles that have a similar structure to that of phospholipid vesicles like liposomes. They can be used to encapsulate aqueous solutes and act as drug and cosmetic carriers. They are formed by the self-assembly of non-ionic surfactants in aqueous media. The application of heat or physical agitation helps niosomes to attain a close bilayer structure (Uchegbu and Vyas, 1998). The hydrophobic parts are shielded from the aqueous solvent while the hydrophilic head groups are in contact with it. The structure is depicted in Figure 4. The lower cost, greater stability and ease of storage of non-ionic surfactants, used in niosomes, made niosomes popularly used in transdermal delivery for drugs and cosmetics.
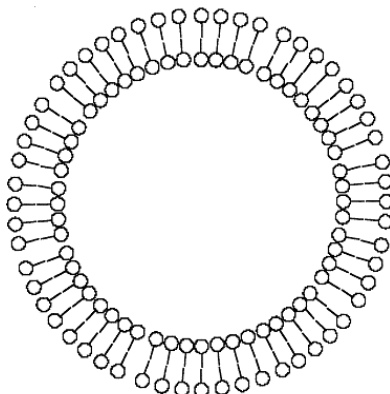
Figure 4. Schematic representation of a niosome , ◯ = hydrophilic head group,
      -- = hydrophobic tail (Uchegbu and Vyas., 1998)

1. Materials in niosome formulation

1.1. Non-ionic surfactant

A surfactant used for preparation of niosomes must have a hydrophilic head and hydrophobic tail. Many non-ionic surfactants form vesicles with difference in toxicity. The alkyl ester surfactants are less toxic than alkyl ether surfactants due to ester-linked surfactant degraded by esterase to triglyceride and fatty acid in vivo (Biswal et al., 2008). The sorbitan ester surfactants are commonly used in cosmetic and pharmaceutical products. The hydrophilic-lipophilic balance (HLB) is a good indicator of the vesicle forming ability of any surfactant. With the sorbitan surfactants, a HLB number of between 4 and 8 was found to be compatible with

vesicle formation (Yoshioka, Sternberg and Florence, 1994; Uchegbu and Florence, 1995). Many niosomal formulations used sorbitan ester (Span), including Span20, Span40, Span60, Span80 and Span85 (Yoshioka et al., 1994; Yoshioka and Florence, 1994; Yoshioka et al., 1995; Hao et al., 2002; Suwakul, Ongpipattanakul and Vardhanbhuti, 2006; Azeem et al., 2008; Kapadia et al., 2009)

1.2. Membrane additives
1.2.1. Cholesterol

Various additives must be included in the formulation in order to obtain stable niosomes. The most common additive found in niosome systems is cholesterol. The mixture of surfactant and cholesterol is popularly used to prepare niosomes. Cholesterol is known to abolish the gel to liquid phase transition of liposome (New, 1990) and niosome systems, resulting in niosome that are less leaky. Most formulations used cholesterol in a 1:1 molar ratio (Uchegbu and Vyas, 1998). However, even after the addition of cholesterol, the intrinsic phase transition behavior of surfactants still influences the properties of the dispersions : notably the membrane permeatibility, encapsulation efficiency, bilayer rigidity.

Basically, niosomes should be stabilized by the addition of a charged molecule to the bilayer such as dicetyl phosphate and stearyl amine (Sahin, 2007). Another additive is a non-ionic substance, poly-24-oxyethylene cholesteryl ether (Solulan C-24). It is added to the formulation to prevent niosomes aggregation (Uchegbu and Vyas, 1998; Arunothayanan, Sooksawate and Florence., 1999). The structure of Solulan C-24 is shown in Figure 5.
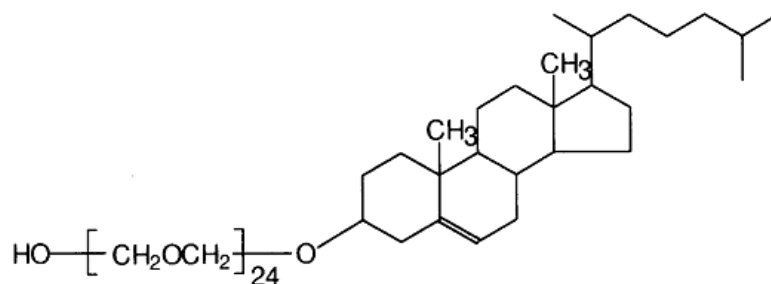
Figure 5. Chemical structure of Solulan C-24 (poly-24-oxyethylene cholesteryl ether) (Uchegbu and Florence, 1995)

1.3. Surfactant and lipid levels

The level of surfactant/lipid used to make niosomal dispersions is generally 10-30 mM. Altering the surfactant:water ratio during hydration step may affect the system microstructure. The increasing of the surfactant/lipid level also increases the total amount of drug encapsulated.

2.   Method for niosome preparation

The formation of vesicular assemblies requires the input of some form of energy and all the experimental methods consist of the hydration of a mixture of the surfactant/lipid at elevated temperature, size reduction to obtain a colloidal dispersion.

2.1. Hand shaking/lipid layer hydration

The mixture of surfactant/lipid is dissolved in organic solvent. Then, the organic solvent is removed by reduced pressure leads formation of drug surfactant/lipid film. The surfactant/lipid film is hydrated with aqueous solution at temperature above the phase transition temperature of surfactant with constant mild shaking (Nasseri and Florence, 2003).

2.2. Reverse phase evaporation

This method provides a high aqueous space-to-lipid ratio and able to entrap a large percentage of the aqueous material. This technique can entrap the aqueous phase up to 65% at low salt concentrations and under optimal conditions and encapsulate even large macromolecules with high efficiency (Lasch, Weissig and Brandl, 2003).

The procedure is based on the formation of invert micelles which small water droplets are stabilized by a phospholipid monolayer and dispersed in an excess of organic solvent. The invert micelles are formed upon sonication of a mixture of a aqueous phase which contains the water soluble molecules to be encapsulated, and organic phase which the amphiphilic phospholipid molecules have been solubilized. The slow removal of organic solvent leads to transformation of these inverted micelles into a viscous gel-like state. At a critical point in this procedure, the gel state collapses and some of the inverted micelles disintegrate. The lipid monolayer which

enclosed the collapses vesicles initiate to adjacent and intact vesicles, formations of the outer leaflet of bilayer of a large unilamellar vesicles (Szoka and Papahadjopoulos, 1978). The schematic diagram of the process are shown in Figure 6. The main drawback of this method is the exposure of the material to be encapsulated to an organic solvent, which may lead to denature some drugs.
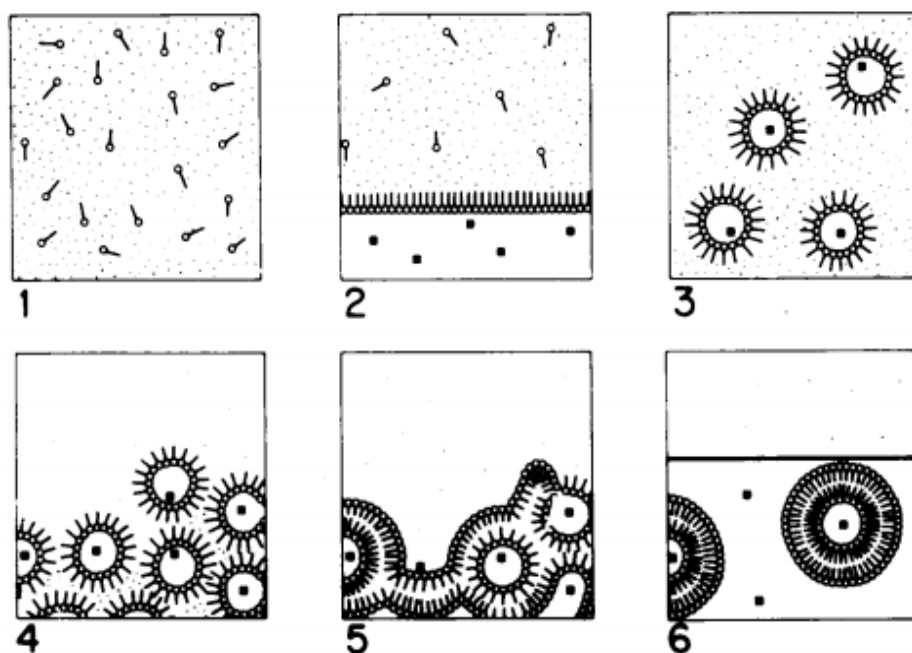
Figure 6. Diagram of the formation of reverse phase evaporation. The lipids are dissolved in organic solvent to form a lipid-in-solvent solution (panel 1). The aqueous phase containing the drug (˙ drug) is added to form a two phase system (panel 2). The small water droplets stabilized by a monolayer, invert micelles (panel 3). Afterward, organic solvent was removed and invert micelles collapsed into a viscous gel-like state (panel 4). The gel state collapsed (panel 5) and the remaining micelles formed vesicles (panel 6). (Szoka and Papahadjopoulos, 1978)

2.3. Ether injection

The surfactant or surfactant-cholesterol or surfactant-cholesterol-drug solution mixture dissolves in organic solution. Then, the organic solution is injected slowly into aqueous solution of drug or aqueous phase which heated above the boiling point of the organic solvent (Baillie et al., 1985).

2.4. Bubbling of inert gas

The homogenization of a surfactant/lipid mixture followed by the bubbling of nitrogen gas through this mixture. Apparently the homogenization step may be omitted from the procedure without affecting particle size (Talsma et al., 1994)

3. Characterization of niosomes

Methods appropriate for investigation and characterization of colloids are frequently used in drug development and may be found in pharmaceutical laboratories. Both macroscopic and microscopic techniques are used.

3.1. Morphology

Polarized light microscopy is suitable for detection of liquid crystals because liquid crystals show birefringence just like real crystals. Each liquid crystal shows typical black and white textures (Muller, 2004). The electron microscopy techniques are used to verify vesicle formation and examine lamellarity and morphology of vesicles. This technique include transmission electron microscopy (TEM) (Guinedi et al., 2005), cryo-TEM (Muller, 2004) and freeze-fraction electron microscope (Yoshioka et al., 1994).

3.2. Partical size and size distribution

The particle size is an important parameter in in-process control and particularly in quality assurance because the physical stability of vesicle dispersions depends on particle size and particle size distribution. The most commonly method to measure the particle size from below 200 nm up to 1 μm is photon correlation spectroscopy (PCS) (Muller, 2004). The dynamic processes in the dispersion such as Brownian molecular motion cause variations in the intensities of the scattered light.

3.3. The separation of entrapped material

The hydration of surfactant and lipid mixtures rarely leads to the entire drug being encapsulated, regardless of the drug loading optimization steps taken. It is thus often a requirement that unencapsulated drug be removed by various means. The

methods that have been used for the removal of unentrapped material are exhaustive dialysis (Baillie et al., 1985; Hao et al., 2002), Ultracentrifugation (Guinedi et al., 2005) and gel filtration (Yoshioka and Florence, 1994).

4. Niosomes as drug delivery system

4.1. Ocular drug delivery system

Vesicular drug delivery system used in ophthalmics such as liposomes and niosomes help in providing prolonged and controlled action at the corneal surface and preventing the metabolism of the drug by enzymes present at the corneal surface (Kaur, Singh and Kanwar, 2000). Niosome in topical ocular delivery are preferred over other vesicular systems because they are chemically stable, low toxicity, biodegradable, biocompatible and non-immunogenic. They can improve the performance of the drug via better availablility and controlled delivery at a particular site (Carafa et al., 1998).

Acetazolamide loaded in niosomes prepared from Span® 60 and cholesterol in a 7:6 molar ratio has higher entrapment efficiency of 32.21% (Guinedi et al., 2005). The evaluation of intraocular pressure (IOP) in New Zealand rabbits of acetazolamide niosomes and acetazolamide solution showed that acetazolamide niosomes have effectively prolong a decrease in the IOP than acetazolamide solution.

4.2. Oral drug delivery system

Niosomes can prolong the circulation of the entrapped drugs because non-ionic surfactant possess better intrinsic targeting potential and propensity. They have been placed on slow release of drug, resulting controlled activity, reduced toxicity, targeting and modification of distribution profile of drugs (Ahuja et al., 2008) and increased the absorption of drugs from the oral ingestion (Attia et al., 2007).

The entrapment efficiency of acyclovir loaded in niosomes consisted of cholesterol, Span 60 and dicetyl phosphate in the molar ratio of 65:60:5, respectively was 11.00%. The release profile was found to follow Higuchi's equation for free acyclovir and niosomal acyclovir. The percentage drug release of niosomal acyclovir

and free acyclovir showed 16% after 1.0 hour and 72% after 0.5 hour, respectively. The release rate of acyclovir from the niosomal dispersion ($K = 0.0293$ mg/h$^{1/2}$) was significantly lower (P<0.001) than free solution ($K = 0.0654$ mg/h$^{1/2}$). The drug release from the free acyclovir began to plateau after 3 hours, whereas, the release from the niosomal dispersion was continued for 6 hours without reaching plateau. The in vivo study revealed that the niosomal dispersion significantly improved the oral bioavailability of acyclovir in rabbits after a single oral dose of 40 mg/kg. The average relative bioavailability of the drug from the niosomal dispersion in relation of the free solution was 2.55, indicating more than 2-fold increase in drug bioavailability. The half-lives of niosomal dispersion was 19.19 hours that longer than free drug solution was 0.819 hours. The niosomal dispersion showed increasing in the mean residence time of acyclovir reflecting sustained release characteristics (Attia et al., 2007).

4.3. Topical drug delivery system

Stratum corneum is a main barrier of many compounds passing through the skin. Several approaches have been developed to weaken the skin barrier. One possibility for increasing the penetration of drugs and many cosmetic chemicals is the use of vesicular system, such as liposomes and niosomes.

The rationale for use of lipid vesicles as topical drug carriers is four-fold. The first, they may serve as organic solvent for the solubilization of poorly soluble drugs, for instance, corticosteroids; as a result, they can applied the thermodynamic activity maximum at higher local drug concentrations. The second, they may serve a local depot for the sustained release of dermally active compounds including antibiotics, corticosteroids or retinoic acid. The third, they have nonionic surfactant that acts as penetration enhancer into the lipid layers of the stratum corneum and epidermis. The fourth, they may serve as rate-limiting membrane barrier for the modulation of systemic absorption, i.e., they may serve as controlled transdermal delivery system (Schreier and Bouwstra, 1994)

The mechanism of drug permeation with niosomes may resemble that of liposomes. One possible reason for niosomes to enhance permeability is their ability to modify stratum corneum structure; the intercellular lipid barrier in the stratum

corneum may become looser and more permeable by niosome treatment. Another reason is altering adsorption and fusion of niosomes with the skin surface, which leads to a high thermodynamic activity gradient of drug at the interface (Choi and Maibach, 2005).

Azeem et al. (2008) reported that permeation of frusemide from niosomes across rat skin was higher than that from niosomes disperse in carbopol gel. The value of transdermal flux for niosomes formulation was 9.2 $\mu g/cm^2/hr$ that greather than carbopol gel formulation (6.4 $\mu g/cm^2/hr$) and the plain drug solution (1.1 $\mu g/cm^2/hr$). The flux of frusemide from niosomal formulation was significantly higher than that of control. The overall flux enhancement observed for frusemide in this study was due to the effect of vesicular system on skin barrier property. One of the mechanism by which niosomes may contribute to transdermal drug delivery may be ascribed to the fusion of vesicles on the surface of the skin which might lead to the establishment of large concentration gradients of the intercalated drug across the skin and hence enhanced skin permeation.

CHAPTER III

MATERIALS AND METHODS

CRUDE DRUGS

1. Dried Fruits of *Phyllanthus emblica* (purchased from a crude drug dispensary in Bangkok)

MATERIALS

1. Absolute Ethanol (Lab Scan Co., Ltd., Thailand)
2. Acetic acid (Merck, Germany, lot no. K33266463)
3. Acetonitrile, HPLC grade (Lab Scan Co., Ltd., Thailand)
4. Alpha arbutin (generous gift from Food & Cosmetic systems Co.,Ltd., lot no. 41378101)
5. Chloroform, AR grade (Labscan Asia, Thailand)
6. Cholesterol (Fluka, Japan, lot no. 423504)
7. 2,2-diphenyl-1-picryhydrazyl (DPPH) (Sigma-Aldrich, Inc., USA., lot no. S43869)
8. Ethyl acetate (Labscan Asia, Thailand)
9. Gallic acid (Sigma-Aldrich, USA, lot no. 398225)
10. Kojic acid (Namsiang Trading Co., Ltd., Thailand, lot no. 040901)
11. L-3,4-dihydroxyphenylalanine (L-dopa) (Fluka, lot no. 97310)
12. L-ascorbic acid (Fluka, lot no. 95210)
13. Methanol, HPLC grade (Lab Scan Co., Ltd., Thailand)
14. Paracetamol BP2007/EP6.0, 99.4% (generous gift from Defence Pharmaceutical Factory, lot no. 0906073)
15. Phosphoric acid (J.T. Baker, USA, lot no. 0206)
16. Poloxamer 407 (BASF, Germany, lot no. WPYF56C)
17. Potassium dihydrogen phosphate (Merck, Germany, lot no. A262673)
18. 2-Propanol, HPLC grade (Lab Scan Co., Ltd., Thailand)
19. Sodium chloride (Merck, Germany, lot no. K34243404)
20. Sodium dihydrogen phosphate (Merck, Germany, lot no. F1327886)
21. Sodium hydroxide (Merck, Germany, lot no. B0119798)

22. Solulan C-24® (generous gift from Chemico intercorporation Co., Ltd., Thailand, lot no. CR5110900)

23. Sorbitan laurate (Span® 20) (NOF CORP., Japan)

24. Sorbitan monopalmitate (Span® 40) (NOF CORP., Japan)

25. Sorbitan stearate (Span® 60) (NOF CORP., Japan)

26. Sorbitan oleate (Span® 80) (NOF CORP., Japan)

27. Tyrosinase from mushroom (Sigma-Aldrich, USA, Lot no. 079K7000)

APPARATUSES

1. Analytical balance (AG 285, Mettler Toledo, Switzerland)

2. Analytical balance (PG403-S, Mettler Toledo, Switzerland)

3. Centrifuge polycarbonate (10.4 ml) (Beckman Instruments, USA)

4. Freeze dryer (Model Heto power dry PL-9000-90 HSC, Jouan Nordic, Thailand)

5. High performance liquid chromatography system

    -Automatic sample injector (SIL-10A, Shimadzu, Japan)

    -Communication bus module (CBM-20A , Shimadzu, Japan)

    -Column (Alltech Alltima C18, 5μm, 150mm x 4.6 mm, lot no.04111942.1)

    -Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)

    -Precolumn (μBondapack C18, 10 μm,125 A°, Water Corporation, Ireland)

    -UV-VIS detector (SPD-10A, Shimadzu, Japan)

6. Inverted microscope (Model IX51, E for L international co., Ltd.)

7. Light microscope (Nikon Eclipse E200, Japan)

8. Micropipette (Biohit, Finland)

9. Microplate reader (Victor®, Perkin Elmer Ltd., USA)

10. Modified Franz diffusion cells (Crown glass, USA)

11. Round bottom flask 1000 ml (Schott Duran, Germany)

12. Refrigerated incubator (FOC 225I, VELP Scientifica, Italy)

13. pH meter (Model 420A, Orion, USA)

14. Rotary evaporator (Rotavapor R-215, Buchi, Switzerland)

15. Sonicator (Transsonic digitals, Elma, Germany)

16. Soxhlet extractor apparatus (N.K. Joshi &co., India)

17. Ultracentrifuge (L80, Beckman, USA)

18. Ultrasonic bath (Transsonic digitals T900/H, Elma, Germany)

19. UV spectrophotometer (Model UV-1601), Shimadzu, Japan)

20. Vacuum Pump (Water model DOA-VI30-VN, Millipore, USA)

21. Vortex mixer (Vortex Genie-2, Scientific Industries, USA)

22. Viscometer (RotoVisco RVI, Germany)

23. Water bath (Model WB 22, Becthai Co., Ltd., Thailand)

24. Zetasizer Nano (Model Nano-ZS, Malvern, England)

Accessories

1. Centrifuge bottle polycarbonate (10.4 ml) (Beckman Instruments, USA)

2. Disposable syringe filter nylon 13 mm, 0.45 µm (Chrom Tech, USA)

3. Parafilm (Pechiney Plastic Packaging, Inc., USA)

4. Porcine skin (donated by Ja-Daeng Farm, Nakornpratom, Thailand)

5. Syringe needle gauge No. 23 (Nipro Corporation, Thailand)

6. Syringe, 3 ml (Nipro Corporation, Thailand)

7. TLC Alumina sheet silica gel 60F 254 (E.Merck, Germany)

8. Whatman filter paper No.1, diameter150 mm (Whatman International Ltd. England)

9. 96-well microplates (Corning, Inc., USA)

METHODS

A. Preparation of Crude Extracts from Fruits of *Phyllanthus emblica*

    1. Preparation of crude drug

Dried fruits of *P.emblica* were purchased from a crude drug dispensary in Bangkok, Thailand. They were ground to coarse powder by a cutter mill.

    2. Extraction process

The dried coarse powder of *P.emblica* (4 kg) was defatted with hexane in Soxhlet extraction apparatus (Figure 7) until the solvent in the thimble was clear. Then, the marc was successively extracted with ethyl acetate until the solvent was clear. Hexane and ethyl acetate extracts were filtered through Whatman no.1 filter paper. The hexane and ethyl acetate extracts were dried under vacuum (Mahattanapokai, 2003). The ethyl acetate extract was dissolved in water and further lyophilized into dry powder. The obtained powder extract used in the whole study was so called the emblica extract and kept in a dry and cool place.
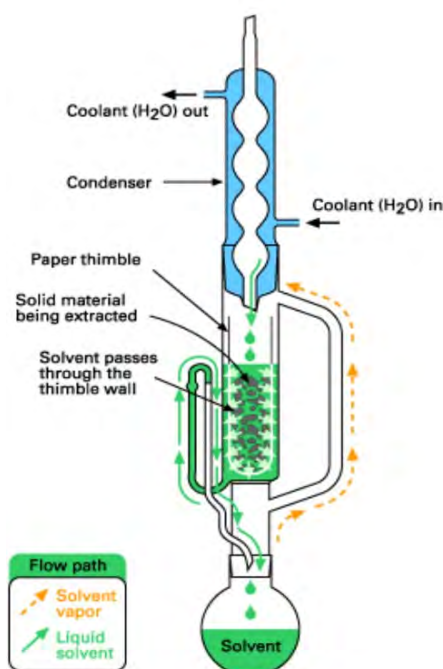
Figure 7. Soxhlet extractor
(http://www.aquaculture.ugent.be/Education/coursematerial/online%20courses/ATA/
analysis/crudprot.htm)

28

Dried Fruits of *Phyllanthus emblica*

Grinding

P.emblica powder

Defatting with hexane

Marc

Ethyl acetate

Hexane solution

Ethyl acetate solution

Evaporating under vacuum

Evaporating under vacuum

Hexane extract

Ethyl acetate extract

Water

Water extract

Lyophilization

Residue

Emblica extract

Figure 8 Schematic diagram of the solvent extraction process of *Phyllanthus emblica* fruits

B. Identification and Determination of Active Constituents from *Phyllanthus emblica*

1. Identification by Thin layer chromatographic (TLC) method

TLC method was used for identification of gallic acid which as a biological in crude extract from *Phyllanthus emblica* (Mahattanapokai, 2003). The hexane and emblica extracts were spotted on TLC Alumina sheet compared with standard solution of gallic acid. The plate was placed into mobile phase, composed of ethyl acetate:methanol:water (15:1.5 :1) inside a closed chamber. The plate was visualized with UV detector at 254 nm. The parameter used to describe is the R_f value (Ganshirt, 1965).

$$R_f \quad = \quad \frac{\text{Distance of spot centre from start point}}{\text{Distance of solvent front from start point}}$$

2. Determination of active constituents by high performance liquid chromatographic (HPLC) method

The gallic acid content of emblica extract was determinated by HPLC method because of its specificity and high sensitivity.

2.1. The chromatographic condition

The chromatographic method was modified from the method reported by Natural Remedies Private Limited.

Column	:	Alltech Alltima C18, 5 µm, 4.6x150 mm
Precolumn	:	µBondapack C18, 10 µm, 125A°
Mobile phase	:	Water:Acetonitrile:Acetic acid (97:3:0.5)
Injection volume	:	10 µl
Flow rate	:	1 ml/min
Detector	:	UV detector at 273 nm
Temperature	:	ambient
Run time	:	15 min
Internal standard	:	Paracetamol

The mobile phase was freshly prepared, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min before use.

2.2. Standard solution

2.2.1. Preparation of internal standard

The stock solution of internal standard was prepared by accurately weighing 5 mg of paracetamol into a 5 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of paracetamol stock solution was 1 mg/ml.

2.2.2. Preparation of standard solutions

The 5 mg part of standard gallic acid was accurately weighed and transferred into a 10 ml volumetric flask, diluted and adjusted to volume with methanol. The stock solution had the final concentration of gallic acid of 500 μg/ml.

The solutions of 40, 80, 120, 160, 200, 240 and 280 μl of standard gallic acid stock solution and 50 μl of internal standard stock solution were added into 5 ml volumetric flasks. The dilution to volume with mobile phase gave final concentrations of 4, 8, 12, 16, 20, 24 and 28 μg/ml of gallic acid, respectively.

2.2.3. Preparation of sample solution

The 10 mg part of emblica extract was accurately weighed and transferred into a 10 ml volumetric flask, diluted and adjusted to volume with methanol. The stock solution had the final concentration of emblica extract of 1000 μg/ml.

The solution of 100 μl of emblica extract stock solution, and 50 μl of internal standard stock solution were added into a 5 ml volumetric flask. The dilution to volume with mobile phase gave a final concentration of 20 μg/ml of emblica extract.

2.3. Validation of HPLC method (USP 29, 2006)

The validation of the HPLC method use in this study was carried out. The parameters of the analysis were determined as specificity, linearity, accuracy and precision.

2.3.1. Specificity

The specificity of the method was determined by comparing the chromatogram of the test solution with that of standard gallic acid solution. Under the chromatographic conditions used, the peak of gallic acid must be completely separated and not be interfered by the internal standard.

2.3.2. Linearity

The linearity was determined from the coefficient of determination (R^2). Three sets of seven concentrations of standard solutions were prepared and analyzed. The linear regression analysis was determined from the plot between the peak area ratio versus their concentrations.

2.3.3. Accuracy

The accuracy of analytical method is closeness of test results obtained by the method to the true value. The accuracy of the method was determined from the percentage of recovery. Five sets of three concentrations (low, medium, high) of gallic acid at 5, 15, 26 µg/ml were prepared and analyzed, respectively. The percentage of recovery for each concentration was calculated from the ratio of inversely estimated concentration to actual concentration mutipled by 100.

2.3.4. Precision

a) Within run precision

The within run precision was determined by analyzed five sets of three concentrations (low, medium, high) at 5, 15, 26 µg/ml in the same day. Peak area ratio of gallic acid was calculated and the percentage of coefficient of variation (%CV) of each concentration was determined.

b) Between run precision

The between run precisions was determined by analyzed three concentrations of gallic acid at 5, 15, 26 µg/ml on five different days. The percent coefficient of variation (%CV) of gallic acid of each concentration was determined.

Acceptance criteria :

For accuracy, the percentage of recovery should be within 98-102% for each nominal concentration, whereas the percentage coefficient of variation for both within run precision and between run precision should be less than 2% .

The percentage amount of gallic acid in emblica extract (w/w) was calculated from the amount obtained from the linear regression equation, divided by the weight of emblica extract used and multiplied with 100.

C. Evaluation of Emblica Extract for Antioxidant Activity by DPPH Method

Hydrogen-donating activity of the emblica extract, standard gallic acid and ascorbic acid were determined using the 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical. Hydrogen-donating activity of an antioxidant was determined based on its ability to donate hydrogen to DPPH radical (Jithavech, 2005).

1. Preparation of 0.1 mM DPPH radical solution

DPPH was accurately weighed of 7.93 mg and dissolved in 200 ml of absolute ethanol. The concentration of the solution obtained was 0.1 mM of DPPH solution.

2. Preparation of test samples

The test samples were prepared with initial concentration of 1 mg/ml. The stock test solution was diluted with absolute ethanol to obtain a suitable range of final concentrations (µg/ml) as shown in Table 3.

Table 3. The initial and final concentrations (µg/ml) of the test samples

Initial concentration (µg/ml)	60	20	10	6	5	3	2	1.5	1	0.5
Final concentration (µg/ml)	30	10	5	3	2.5	1.5	1	0.75	0.5	0.25

3. Measurement of activity

An ethanolic solution (1 ml) of the sample at various concentrations (0.25-30 µg/ml) were added with 1 ml of DPPH (0.1 mM) solution. The mixture was mixed well and left to stand at ambient temperature for 30 min in the darkness. The absorbance was measured by a UV-Vis spectrophotometer at 517 nm. Gallic acid was used as standard, whereas ascorbic acid was compared as a positive control and absolute ethanol as a negative control (Mahattanapokai, 2003). Assay of the mixture at each concentration was performed in triplicate. The ability to scavenge the DPPH radical was calculated using the follow equation.

$$\% \text{ inhibition} = \frac{(\text{Abs. control} - \text{Abs. sample}) \times 100}{\text{Abs. control}}$$

Abs control : The absorbance of mixture containing 1 ml of 0.1 mM DPPH solution and 1 ml of absolute ethanol without test sample

Abs sample : The absorbance of mixture containing 1 ml of 0.1 mM DPPH solution and 1 ml of test sample solution

4. Calculation of IC_{50}

After calculation of % inhibition for each concentration of the test sample, a relationship between % inhibition and concentration was plotted. The concentration at 50% inhibition (IC_{50}) of each test sample was calculated from the linear regression equation of the initial linear portion of the graph.

D. Evaluation of Emblica Extract for Antityrosinase Activity (Jithavech, 2005)

Tyrosinase inhibition activity was performed in a 96-well microplate reader (VICTOR$^{®}$) with 492 nm interference filter used for detection. The reaction mixture was monitored by measuring the change in absorbance at 492 nm due to formation of the dopachrome for 10 min. Dopachrome is one of the intermediate substances in the melanin synthesis. The potential tyrosinase inhibitor will cause a decrease in dopachrome absorption.

1. Preparation of reaction mixture

1.1. Preparation of phosphate buffer (pH 6.8)

Solution A : 20 mM $NaH_2PO_4.2H_2O$ (312 mg) was dissolved in 100 ml of water

Solution B : 20 mM Na_2HPO4 (284 mg) was dissolved in 100 ml of water

Then, solution A (70 ml) and B (30 ml) were mixed to provide phosphate buffer pH 6.8.

1.2. Preparation of 0.85 mM L-DOPA

L-DOPA 0.80 mg was dissolved in 5 ml phosphate buffer pH 6.8 to yield 0.85 mM solution.

1.3. Preparation of 480 IU/ml mushroom tyrosinase solution

Mushroom tyrosinase enzyme 0.610 mg (label activity 3933 IU/mg) was dissolved in 5 ml phosphate buffer pH 6.8 to yield 480 IU/ml enzyme solution.

1.4. Preparation of test samples

The test samples were prepared with initial concentration of 10 mg/ml. The stock test solution was diluted with water to obtain a suitable range of concentration (0.5-60 µg/ml).

2. Measurement of activity

The absorbance of the reaction mixture was measured in 4 wells (A, B, C and D). The substance was added in the order of mixing as follows :

A (Control)   40 µl of phosphate buffer pH 6.8

80 µl of water

40 µl of mushroom tyrosinase solution 480 IU/ml

B (Blank of A) 80 µl of phosphate buffer pH 6.8

80 µl of water

C (Sample)   40 µl of phosphate buffer pH 6.8

80 µl of sample solution in water

40 µl of mushroom tyrosinase solution 480 IU/ml

D (Blank of C) 80 µl of phosphate buffer pH 6.8

80 µl of sample solution in water

After each well was mixed and preincubated at room temperature for 10 minutes, 40 µl of 0.85 mM L-DOPA was added, and the mixture was further incubated at room temperature for 10 minutes. The absorbance of each well was measured at 492 nm with the microplate reader. Gallic acid was used as a standard, whereas kojic acid and alpha arbutin were compared as positive controls. Assay of the mixture at each concentration was performed in triplicate.

3. Calculation of the percent inhibition of tyrosinase enzyme

The percent inhibition of tyrosinase activity was calculated from the equation as follows:

% Tyrosinase inhibition = $\dfrac{[\,(A\text{-}B) - (C\text{-}D)\,] \times 100}{(A\text{-}B)}$

A : The absorbance after incubation at 492 nm without test sample

B : The absorbance after incubation at 492 nm without test sample and
enzyme

C : The absorbance after incubation at 492 nm with test sample

B : The absorbance after incubation at 492 nm with test sample but
without enzyme

4. Calculation of IC$_{50}$

After calculation of % tyrosinase inhibition for each concentration of the test sample, a relationship between % tyrosinase inhibition and concentration was plotted. The concentration at 50% inhibition (IC$_{50}$) of each test sample was determined by extrapolation of the plot between % tyrosinase inhibition and concentration.

E. Preparation and Characterization of Niosomes

1. Preparation of niosomes (Szoka and Papahadjopoulos,1978 ; New, 1990 and Lasch et al., 2003)

Niosomes were prepared by reverse phase evaporation and verified in types of surfactant and surfactant to cholesterol ratios. The reverse phase evaporation technique was able to entrap a large percentage of the aqueous material presented.

The total lipid concentration used was 60 µmol/ml of aqueous phase. Cholesterol, surfactant and Solulan C-24 were calculated to get final lipid concentration in 150 ml of final volume of niosome preparation.

Firstly, varied amounts of cholesterol, surfactant and Solulan C-24 (Table 4) were accurately weighed and dissolved with chloroform in a 1000 ml round bottom flask. Secondly, either a predetermined volume of ultrapure water or 0.5% w/v emblica extract in ultrapure water was prepared. Afterward, the aqueous phase was introduced into the lipid solution by rapidly injecting through a needle guage No.23 from a 20 ml syringe. The system was tightly closed and sonicated for 5 min in bath sonicator at temperature 8 ℃. Thirdly, the flask was assembled directly to the rotary evaporator and the contents were evaporated at 60 ℃ under reduced pressure until gel forming. Fourthly, the flask was removed out and was subjected to vigorous agitation by vortex mixer until gel collapsed. Afterwards, traces of solvent were removed under reduced pressure. Finally, size reduction was carried out by sonication at 60 ℃ for 60 min.

Table 4. Compositions of lipid in formulations in various ratios of cholesterol and surfactant*.

Surfactant	Surfactant : cholesterol ratio	Ratio of surfactant : choleserol : Solulan C-24 (molar ratio)
Span 20	1:1	47.5 : 47.5 : 5
	6:4	57 : 38 : 5
	7:3	66.5 : 28.5 : 5
Span 40	1:1	47.5 : 47.5 : 5
	6:4	57 : 38 : 5
	7:3	66.5 : 28.5 : 5
Span 60	1:1	47.5 : 47.5 : 5
	6:4	57 : 38 : 5
	7:3	66.5 : 28.5 : 5
Span 80	1:1	47.5 : 47.5 : 5
	6:4	57 : 38 : 5
	7:3	66.5 : 28.5 : 5

*Each formulation was fixed with 5% mole of Solulan C-24 and prepared in triplicate.

2. Characterization of niosomes

The characteristics of niosomes of each formulation such as appearance, size and size distribution and the percentage of entrapment efficiency were investigated.

2.1. Microscopic appearances

The microscopic features, such as shape, size and cross polarized light of niosomes were observed. They were photographed under the microscope magnification of 10×40.

2.2. Particle size analysis

Particle size analysis was performed by photon correlation spectroscopy (PCS), (Malvern, UK) at 25 ºC. A sample was dispersed, diluted in ultrapure water and put in a quart cuvette, which was placed into the instrument. PCS yields the mean particle size and the polydispersity index (Pdi) as the measure of size distribution. Each obtained value from each formulation was the average of three measurements and three replicates.

2.3. Entrapment efficiency

The determination of entrapped gallic acid amount was performed by HPLC method due to its specificity and high sensitivity.

2.3.1. Assay in the entrapment study

2.3.1.1. The chromatographic condition

The chromatographic method was modified from the method reported by Natural Remedies Privated Limited.

Column	:	Alltech Alltima C18, 5 µm, 4.6x150 mm
Precolumn	:	µBondapack C18, 10 µm, 125Aº
Mobile phase	:	Water:Acetonitrile:Acetic acid (97:3:0.5)
Injection volume	:	10 µl
Flow rate	:	1 ml/min
Detector	:	UV detector at 273 nm
Temperature	:	ambient
Run time	:	15 min
Internal standard	:	Paracetamol

The mobile phase was freshly prepared, filtered through 0.45 µm membrane filter and then degassed by sonication for 30 min before use.

2.3.1.2. Standard solution

a) Preparation of internal standard

The stock solution of internal standard was prepared by accurately weighing 5 mg of paracetamol into a 5 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of paracetamol stock solution was 1 mg/ml.

b) Preparation of standard solutions

The 100 mg part of standard gallic acid was accurately weighed and transferred into a 10 ml volumetric flask, diluted and adjusted to volume with isopropanol. The stock solution had the final concentration of gallic acid of 10 mg/ml.

The 0.4 ml part of standard gallic acid stock solution was transferred into a 10 ml volumetric flask, diluted and adjust to volume with methanol. The stock solution had the final concentration of gallic acid of 400 µg/ml.

The aliquot solutions of 4, 8, 20, 40, 60 and 80 µl of standard gallic acid in methanol and 10 µl of internal standard stock solution were added into 10 ml volumetric flasks. The dilution to volume with mobile phase gave final concentrations of 0.16, 0.32, 0.80, 1.6, 2.4 and 3.2 µg/ml of gallic acid, respectively.

2.3.1.3. Validation of HPLC method

The validation of HPLC method was carried out by analysis of its specificity, linearity, accuracy and precision.

a) Specificity

The specificity of the method was determined by comparing the chromatogram of the test solution with that of gallic acid standard solution. Under the chromatographic conditions used, the peak of gallic acid must be completely separated and not be interfered by the peaks of isopropanol, methanol, paracetamol, and compositions of niosome.

b) Linearity

The linearity was determined from the coefficient of determination (R^2). Three sets of six concentrations of standard solutions were prepared and analyzed. The linear regression analysis of the peak area ratios versus their concentrations was performed.

c) Accuracy

The accuracy of analytical method is closeness to test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Three sets of three concentration (low, medium, high) of gallic acid solutions at 0.24, 1.20, 2.80 µg/ml were prepared and analyzed, respectively. Additionally, a blank niosome formulation and a blank niosome serum were spiked with gallic acid stock solution to attain niosome and niosome serum with gallic acid concentration at 0.24, 1.20, 2.80 µg/ml. The percentage of recovery for each concentration in three systems were calculated from the ratio of inversely estimated concentration to actual concentration mutipled by 100.

d) Precision

d1) Within run precision

The within run precision was determined by analyzed three sets of three concentrations (low, medium, high) at 0.24, 1.20, 2.80 µg/ml in the

same day. Peak area ratio of gallic acid was calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

d2) Between run precision

The between run precisions was determined by analyzed three concentrations of gallic acid at 5, 15, 26 µg/ml on five different days. The percent coefficient of variation (%CV) of gallic acid of each concentration was determined.

Acceptance criteria :

For accuracy, the percentage of recovery should be within 98-102% for each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2% .

2.3.2. Entrapment efficiency of gallic acid loaded in niosomes

The niosomal dispersions were weighed into balanced centrifuge tubes. The encapsulated vesicle were separated after ultracentrifugation at 65,000 rpm at 4 °C for 8 hours. The sedimented pellets were lyzed by isopropanol. After sonicated, the clear solution obtained was diluted and adjusted to volume with methanol. The solution of pellets in methanol was diluted and adjusted with mobile phase and assayed by HPLC method. The entrapment efficiency was calculated by the equations below (Kongaimpitee, 2007).

$$\text{Entrapment efficiency (\%)} = \frac{\text{Amount of gallic acid encapsulated} \times 100}{\text{Total loading amount}}$$

$$\text{\% Recovery} = \frac{(A + B) \times 100}{\text{Total loading amount}}$$

Where A is amount of gallic acid entrapped in niosomes, B is amount of free gallic acid in supernatant.

2.4. Stability of niosomes containing emblica extract

2.4.1. Physical stability

Physical stability of niosomes containing emblica extract were investigated by observing any change of physical characteristics as described in topic E2 and storage in a refrigerator incubator at 4 ºC and 30 ºC for three months.

2.4.2. Chemical stability

Chemical stability of niosomes containing emblica extract were

investigated in tight container and protected from light after storage in a refrigerator incubator at 4 ºC and at 30 ºC for three months. The triplicated of each system were performed.

Quantitations of active component, gallic acid were determined by HPLC method as described in topic F3.

F. Preparation of Niosome Serums

1. Formulation of niosome serums

Serum formulation was formulated by using poloxamer 407. From the preliminary study with 30-40% of poloxamer 407, it was found that 32% w/v was appropriate. The mixing volume ratio between viscous gel and niosome dispersion was 1:1 that made the concentration of poloxamer 407 to be 16% w/v. Consequently, the physical appearances such as color, clarity, pH and viscosity, were investigated.

2. Characterization of niosome serums

Before physicochemical characterization, all dispersion systems containing niosomes were prepared and stored at room temperature for 1 day for equilibrium.

2.1. pH

The measurement of pH value was performed by pH-meter (Sartorius, USA). Each obtained value from each formulation was the average of 3 replicates.

2.2. Viscosity

Viscosity measurement was performed by viscometer (RotoVisco V1, Germany) at 30±2ºC. The cone C35/1º Ti was selected in the experiment. Each obtained value from each formulation was the average of 3 replicates.

2.3. Stability of viscous dispersion system containing niosomes

2.3.1. Physical stability

Physical stability of dispersion system containing niosomes was carried out in the same manner as described in topic F.2 after storage at 4 º C and 30 ºC for 3 months.

2.3.2. Chemical stability

Chemical stability of viscous dispersion system containing niosomes was carried out in the same manner as described in topic E.2 after storage at 4 º C and 30 ºC for 3 months.

G. In vitro Skin Permeation Study of Niosomes Loaded with Emblica Extract and Serum

The preparation of a full-thickness abdominal skin membrane was performed by the following step. Firstly, subcutaneous fat and extraneous tissues were removed by using scissors and forceps. Afterward, separated skin was then cleaned and bathed in purified water. Finally, individual sheet of clean separated skin was wrapped in aluminium foil and stored in a freezer at -20 ºC and the skin was thawed under room temperature 1 hour before use.

The in vitro permeation study was performed by using modified Franz diffusion cells, with a newborn pig abdominal skin. The porcine skin was soaked in receptor solution for 1 hour before use. Then, the skin was clamped between the donor and receptor compartments of the cells. The receptor solution was phosphate buffer saline pH 7.4, and it was maintained at 37±1ºC using a thermostatic water bath and magnetically stirred at 600 rpm throughout the experiment. The receptor solution and the skin were equilibrated to the desired temperature for 1 hour before the experiment.

After equilibration, the donor compartment was filled with either 500 μl of emblica extract loaded niosomes or 1 gram of viscous dispersion system containing niosomes. The emblica extract solution of 0.5% w/w was used as control. An aliquot of 1 ml sample from the receptor compartment at certain time intervals of 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 and 24 hours. The receptor compartment was replaced with receptor solution to keep the constant volume during the experiment. The samples were analyzed for the amount of gallic acid permeated through the skin into the receptor compartment by HPLC method as mentioned in topic G1.2.

Additionally, at the end of study, the remaining portion of the loaded formulation in the donor compartment were removed. The porcine skin was lyzed and extracted with methanol. The methanol extract was analyzed for gallic acid by HPLC method that as mentioned in topic G1.3. The examination was performed in triplicate. Then cumulative amount of gallic acid through the porcine skin with versus time was plotted from the data obtained from all system studied.

1. The analysis of gallic acid in permeation study

The determination of gallic acid content was performed by HPLC method.

1.1. The chromatographic condition

The chromatographic method was modified from the method reported by Natural Remedies Private Limited.

Column	:	Alltech Alltima C18, 5 μm, 4.6x150 mm
Precolumn	:	μBondapack C18, 10 μm, 125A°
Mobile phase	:	Water:Acetonitrile:Acetic acid (97:3:0.5)
Injection volume	:	20 μl
Flow rate	:	1 ml/min
Detector	:	UV detector at 273 nm
Temperature	:	ambient
Run time	:	15 min
Internal standard	:	Paracetamol

The mobile phase was freshly prepared, filtered through 0.45 μm membrane filter and then degassed by sonication for 30 min before use.

1.2. The determination of gallic acid in PBS buffer pH 7.4

The determination of gallic acid content in PBS buffer pH 7.4 was performed for analysis of gallic acid in receiver medium of Franz cell in permeation study.

1.2.1 Preparation of internal standard solution

The stock solution of internal standard was prepared by accurately weighing 5 mg of paracetamol into a 5 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of paracetamol stock solution was 1 mg/ml.

1.2.2 Preparation of standard solutions

The 10 mg part of standard gallic acid was accurately weighed and transferred into a 25 ml volumetric flask, diluted and adjusted to volume with methanol. The stock solution had the final concentration of gallic acid of 400 μg/ml.

The aliquot solution of 5, 10, 25, 125, 500 and 1,000 μl of standard gallic acid in methanol and 15 μl of internal standard stock solution were added into 25 ml volumetric flasks. The dilution to volume with phosphate buffer saline pH 7.4 gave final concentrations of 0.08, 0.16, 0.40, 2.0, 8.0 and 16 μg/ml of gallic acid, respectively.

1.2.3 Validation of HPLC method

The validation of HPLC method was carried out by analysis of its

specificity, linearity, accuracy and precision.

a) Specificity

The specificity of the method was determined by comparing the chromatogram of the test solution with that of gallic acid standard solution in PBS buffer pH 7.4. Under the chromatographic conditions used, the peak of gallic acid must be completely separated and not be interfered by the peaks of methanol and paracetamol.

b) Linearity

The linearity was determined from the coefficient of determination (R^2). Six concentrations of standard solutions and three replicated were prepared and analyzed. The linear regression analysis of the peak area ratios versus their concentrations was performed.

c) Accuracy

The accuracy of analytical method is closeness to test results obtained by that method to the true value. The accuracy of the method was determined from the percentage of recovery. Three sets of three concentration (low, medium, high) of gallic acid at 0.24, 0.80, 12.0 µg/ml were prepared and analyzed, respectively. The percentage of recovery for each concentration was calculated from the ratio of inversely estimated concentration to actual concentration mutiplied by 100

d) Precision

d1) Within run precision

The within run precision was determined by analyzed three sets of three concentrations (low, medium, high) at 0.24, 0.80, 12.0 µg/ml in the same day. Peak area ratio of gallic acid was calculated and the percent of coefficient of variation (%CV) of each concentration was determined.

d2) Between run precision

The between run precision was determined by analyzed three concentrations of gallic acid at 0.24, 0.80, 12. µg/ml on five different days. The percent coefficient of variation (%CV) of gallic acid of each concentration was determined.

Acceptance criteria :

For accuracy**,** the percentage of recovery should be within 98-102% for each nominal concentration, whereas the percent coefficient of variation for both within run precision and between run precision should be less than 2% .

1.3. The determination of gallic acid in methanol extract

The determination of gallic acid content in methanol extract was performed for analysis of gallic acid in newborn pig skin.

1.3.1 Preparation of internal standard solution

The stock solution of internal standard was prepared by accurately weighed 5 mg of paracetamol into a 5 ml volumetric flask, diluted and adjusted to volume with methanol. The final concentration of paracetamol stock solution was 1 mg/ml.

1.3.2 Preparation of standard solutions

The 10 mg part of standard gallic acid was accurately weighed and transferred into a 25 ml volumetric flask, diluted and adjusted to volume with methanol. The stock solution had the final concentration of gallic acid of 400 μg/ml.

The aliquot solution of 5, 10, 25, 125, 500 and 1,000 μl of standard gallic acid in methanol and 15 μl of internal standard stock solution were added into 25 ml volumetric flasks. The dilution to volume with methanol gave final concentrations of 0.08, 0.16, 0.40, 2.0, 8.0 and 16 μg/ml of gallic acid, respectively.

1.3.3 Validation of HPLC method

The validation of HPLC method was carried out by analysis of its specificity, linearity, accuracy and precision.

The validation procedure was performed in the same manner as described in topic G 1.2

H. Statistic Analysis

The data of % DPPH inhibitor, % tyrosinase inhibitor, % entrapment efficiency, stability study and rate constant of permeation were analyzed by statistically using one-way analysis of variance (ANOVA). When a significant difference ($p<0.05$) was indicated, the data were subjected to multiple comparision by Tukey's test to compare the difference. The statistical package for the social sciences (SPSS) program version 13.0 was used in this study.

CHAPTER IV

RESULTS AND DISCUSSION

A. Preparation of Crude Extracts from Fruits of *Phyllanthus emblica*

Extraction of *P.emblica* has been practically done by using variety of solvents and methods. Many investigations reported the extraction from both dried *P.emblica* fruits and fresh juice of *P.emblica* fruits. A variety of solvents reported included methanol, ethanol, acetone, ethyl acetate, etc. The ethyl acetate extract of *P.emblica* had been reported to exhibit the highest DPPH scavenging activity compared with the hexane and methanol extract (Mahattanapokai, 2003). Similarly, the ethyl acetate extract of *P.emblica* had been reported to exhibit the highest antityrosinase activity compared with the acetone and ethanol extract (Jithavech, 2005).

In this study, fractional extraction was used for the extraction of *P.emblica* dry fruits. The hexane extract of *P.emblica* was obtained by using Soxhlet extractor of 4 kg of *P.emblica* coarse powder for approximately 16 hours for removal of nonpolar compounds, including volatile oils. The ethyl acetate extract of *P.emblica* was obtained by further using Soxhlet extractor by extraction of the marc from hexane extraction with ethyl aceate for approximately 40 hours. The ethyl acetate extract was finally dissolved and extracted with water. The aqueous extract was lyophilized to yield dry powder; which was called emblica extract powder. Only a trace residue of ethyl acetate extract was remained insoluble in water and could not be collected.

The hexane extract was sticky and viscous mass with dark brown color (Figure 9), whereas, the ethyl acetate extract was dark brown sticky solid mass (Figure 10). The lyophilized emblica extract was obtained as light brown powder (Figure 11).

The percentage yields of all *P.emblica* extracts obtained are shown in Table 5.

Figure 9. The hexane extract   Figure 10. The ethyl acetate extract

Figure 11. The emblica extract obtained as lyophilized powder

Table 5. The percentage yields of crude extracts obtained from fractional extraction of *P.emblica* dried fruits powder 4033.4 grams.

Crude extract	Weight of crude extract (g)	% Yield
Hexane extract	29.926	0.742
Ethyl acetate extract	148.403	3.679
Emblica extract (lyophilized powder)	101.297	2.528

The emblica extract gave the percentage yield of 2.528%, whereas the ethyl acetate extract and hexane extract were 3.679 and 0.742%, respectively. The percentage yield obtained was higher than that reported by Kongaimpitee (2007) that the percentage yield of spray dried powder from fresh juice was 1.28%. In this study, the method of extraction yield the emblica extract as powder, which was easy to handle and more acceptable in formulation.

B. **Identification and Determination of Active Constituents from *Phyllanthus emblica***

1. **Identification of gallic acid in crude extracts by thin layer chromatographic (TLC) method**

TLC is the most versatile and flexible chromatographic method. It is rapid and available for a number of samples to be identified. Samples and standard can be applied in a single plate and separated at the same time.

The compound of crude extract was identified by TLC using mixture of ethyl acetate: methanol: water (15:1.5:1) as a developing solvent. The chromatogram of the extracts were compared to the standard gallic acid. From chromatogram in Figure 12, the R_f values, which were the ratio of the distance of sample to the distance of mobile phase on TLC sheet, were compared. It was found that the R_f value of the emblica extract was equal to the standard gallic acid at the value of 0.54, whereas the R_f value of hexane extract was higher at 0.66. From the data obtained, it was confirmed that the emblica extract had the same characteristics as gallic acid, or in other words, was composed of gallic acid as its main constituent. It was shown that the hexane extract had no gallic acid content.

Figure 12. TLC chromatograms of standard gallic acid (G), the hexane extract (H) and the emblica extract (lyophilized powder) (W).

2.   Determination of gallic acid content by HPLC method

Determination of gallic acid content of the emblica extract was performed by HPLC method because of its specificity and high sensitivity.

The validation of analytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

2.1. Specificity

The standard gallic acid had its retention time at 4.528 min, whereas emblica extract showed retention times at 4.553 min. Paracetamol was chosen to be the internal standard since its peak was completely separated from gallic acid peak at retention time of 9.347 min as shown in Figure 13. The results revealed that the chromatographic conditions used were suitable and gave appropriate specificity.

2.2. Linearity

Data of the standard gallic acid concentration and peak area ratio are shown in Table 6. The plot of calibration curve is depicted in Figure 14. The plot showed good linearity with R^2 of 0.9998, which was very close to 1.

2.3. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. Accuracy is calculated as percent recovery by the assay of known added amount of analyses. The percentages of analytical recovery of gallic acid solution are shown in Table 7. The percentages analytical recovery of gallic acid was in range of 100±2% of which indicated that this mehod could be used for analysis in all concentrations studies with high accuracy (USP 29, 2006).

2.4. Precision

The precision of gallic acid analyzed by HPLC method were determined as both within run precision and between run precisions as illustrated in Tables 8 and 9. The coefficients of variation values were 0.62-1.23% and 0.89-1.05%, respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the HPLC method was precise for quantitative analysis of gallic acid in the range studied (USP 29, 2006).
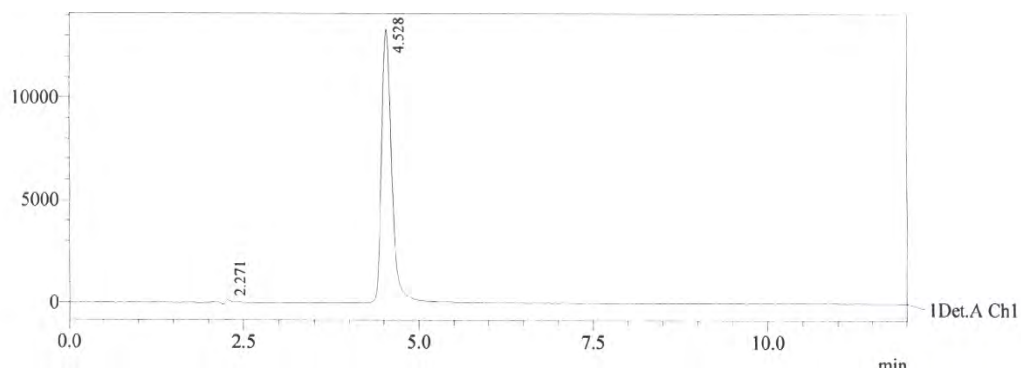
(a) Standard gallic acid (4 µg/ml)
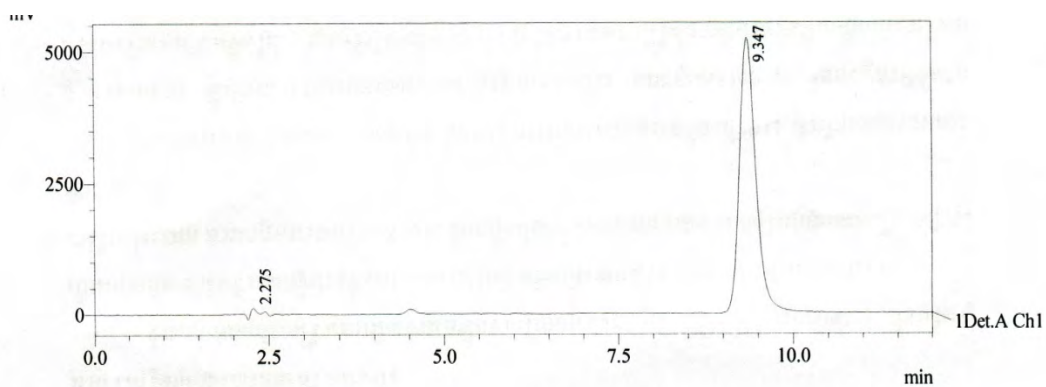
(b) Paracetamol (10 µg/ml)
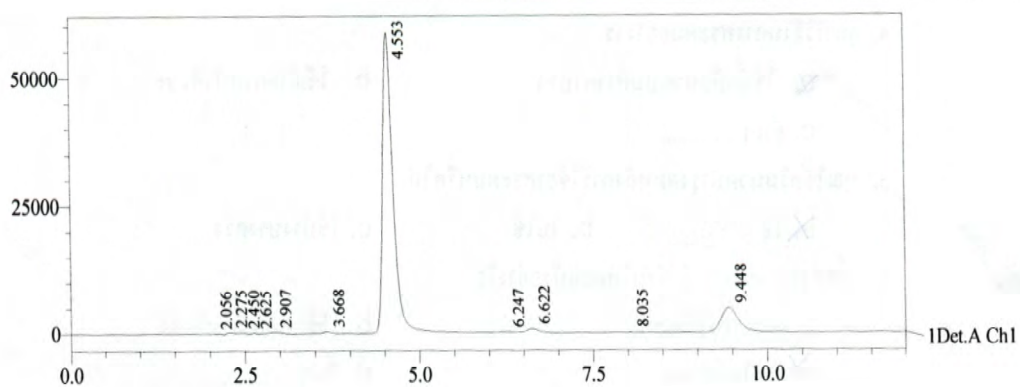
(c) The emblica extract solution with paracetamol

(41.8 µg/ml)

Figure 13 HPLC chromatograms of (a) standard gallic acid solution, (b) paracetamol solution, (c) the emblica extract solution with paracetamol

Table 6 Data for calibration curve of gallic acid in methanol by HPLC method

Concentration (µg/ml)	Peak area ratio			Mean	SD	%CV
	Set 1	Set 2	Set 3			
4	1.0814	1.1042	1.0718	1.0858	0.0167	1.53
8	2.2616	2.2620	2.2404	2.2547	0.0124	0.55
12	3.4267	3.3600	3.3608	3.3825	0.0383	1.13
16	4.6667	4.6102	4.6621	4.6463	0.0315	0.68
20	5.3790	5.7100	5.8219	5.7303	0.0963	1.68
24	6.9785	6.8910	7.0867	6.9715	0.1134	1.63
28	8.2361	8.2800	8.1862	8.2341	0.0470	0.57
R^2	0.9997	0.9990	0.9997	0.9998	-	-
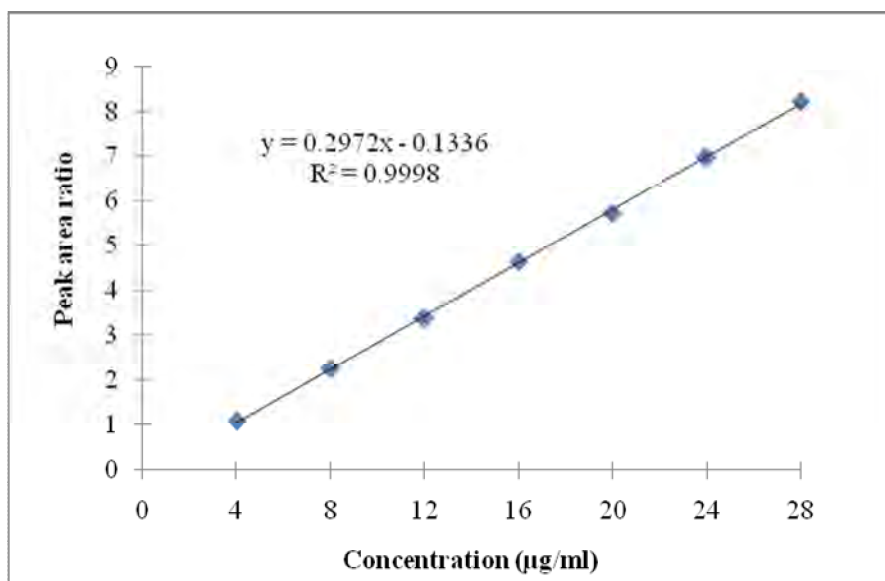
Figure 14 Calibration curve of gallic acid in methanol and mobile phase by HPLC method

Table 7 The percentages of HPLC analytical recovery of low, medium and high concentrations of gallic acid in methanol and diluted with mobile phase by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
5	5.0551	101.10	
5	4.9759	99.52	
5	4.9048	98.10	99.54 ± 1.23
5	5.0172	100.34	
5	4.9314	98.63	
15	15.0870	100.59	
15	14.9549	100.58	
15	15.0659	99.70	100.10 ± 0.62
15	14.8794	100.44	
15	15.0151	99.20	
26	25.8203	99.31	
26	26.0500	100.19	
26	26.3900	101.50	100.50 ± 0.83
26	26.1355	100.52	
26	26.2500	100.96	

Table 8 Data of within run precision of gallic acid in methanol diluted with mobile phase by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)					Mean (µg/ml)	SD	%CV
	1	2	3	4	5			
5	5.0551	4.9759	4.9048	5.0172	4.9314	4.9769	0.0613	1.23
15	15.0883	15.0870	14.9549	15.0659	14.8794	15.0151	0.0937	0.62
26	25.8203	26.0502	26.3901	26.1355	26.2500	26.1291	0.2147	0.82

Table 9 Data of between run precision of gallic acid in methanol and mobile phase by HPLC method

Concentration (μg/ml)	Estimated concentration (μg/ml)			Mean (μg/ml)	SD	%CV
	1	2	3			
5	5.0551	4.9700	4.9895	5.0049	0.0446	0.89
15	15.0883	15.2900	15.0116	15.1300	0.1438	0.95
26	25.8203	25.5890	26.1288	25.8460	0.2708	1.05

In conclusion, the analysis of gallic acid by HPLC method developed in this study showed good specificity, linearity, accuracy and precision.

From the validated HPLC method, it was found that percentage gallic acid contents of emblica extract (lyophilized powder) was $56.29 \pm 2.33\%$. As reported by Kongaimpitee (2007) the gallic acid content in spray dried powder was 32.00% which was lower than that obtained from this study.

C. Evaluation of Emblica Extract for Antioxidant Activity by DPPH Method

Various evaluation methods for antioxidant activity have been used to monitor and compare the antioxidant activity of plant extract. In recent years, oxygen radical absorbance capacity assays have been used to evaluate antioxidant activity.

Antioxidant activity of emblica extract was determined on the basis of hydrogen-donating activity (DPPH radical scavenging activity). The DPPH method was the most common and gave reliable results (Luo et al., 2009; Krishnaiah et al., 2010). This study used the method for evaluation of antioxidant activity on the basis of hydrogen-donating activity (DPPH radical scavenging activity).

The diphenylpicrylhydrazyl (DPPH) method is a simple colorimetry assay of antioxidant activity based on the decrease in absorbance at 517 nm. The addition of antioxidant (RH) results in the changing of the DPPH radical from deep purple color to yellow. The reaction of DPPH and its reduction product by antioxidant is shown as follows:

$$DPPH^\bullet + RH \longrightarrow DPPH\text{-}H + R^\bullet$$

(purple)   (yellow)

The role of an antioxidants is to remove free radicals. One mechanism through which this is achieved, is by donating hydrogen to a free radical in its reduction to a non-reactive species. The hydrogen-donating activity of emblica extract as well as gallic acid and ascorbic acid were determined in terms of their DPPH scavenging activities. The degree of DPPH discoloration is attributed to the hydrogen donating potential of the test compounds. The change in color of DPPH, measured at 517 nm, was compared with a control sample without test extract. The DPPH method measured the antioxidative activity of any compounds on basis of the decrease of absorbance at 517 nm. The antioxidant which has hydrogen donating activity resulted in the change of purple to yellow color (Nikhat, Satynarayana and Subhramanyam, 2009). Ascorbic acid was a potent antioxidant and was widely used to compare the antioxidant activity. Luo et al. (2009) used ascorbic acid as a positive control and standard gallic acid in the study. The result indicated that emblica extract, ascorbic acid and gallic acid had free radical scavenging activities.

The plot of percentages of DPPH scavenging activity (% inhibition) at various concentrations (0.25, 0.5, 0.75, 1, 1.5, 2.5, 3, 5, 10 and 30 µg/ml) of the test samples are shown in Figure 15. The result showed that scavenging effect increased with increasing concentration of test compounds and the plot reached plateau, at which the inhibition became steady at approximately 90% inhibition. The DPPH-scavenging activity of gallic acid exhibited the highest scavenging activity. The relationship between inhibition and concentration of individual antioxidants is shown in Figure 16.

The concentration at 50% inhibiton (IC_{50}) of each sample was calculated from the equation of the linear regression plot and given in Table 10. The linear regression equation for determining the IC_{50} and the coefficient of determination (R^2) are also provided for the individual antioxidant in Figures C1-C3 (Appendix C). The estimated IC_{50} values could be ranked from the lowest to the highest, as follows: gallic acid (0.74±0.10 µg/ml), the emblica extract (1.38±0.14 µg/ml) and ascorbic acid (2.09±0.06 µg/ml). The results exhibited that standard gallic acid had the highest antioxidant potency. The result was in agreement with the publications of Kumaran and Karunakaran (2006) and Kumar et al (2006), who also identified by nitric oxide radical scavenging activity that gallic acid was among the major phenolic acids of the emblica fruit. The finding data was simliar to the result reported by Mahattanapokai

(2003) and Luo et al. (2009) that the DPPH scavenging activity of ethyl acetate extract was higher than that of ascorbic acid.

The IC_{50} data were subsequently analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Tukey method. The results are depicted in Tables C5 and C6 (Appendix C), respectively. It revealed that there was a significant difference among the antioxidants studied ($p < 0.05$). According to Tukey method (Table C7 (Appendix C)), the antioxidants could be ranked regarding their abilities to inhibit or scavenging DPPH radicals. The antioxidant activity ranking is as follows:

<div align="center">Gallic acid > Emblica extract > Ascorbic acid</div>

The IC_{50} ranking were similar to Luo et al (2009) who reported that the DPPH scavenging activities of gallic acid (IC_{50} = 3.18 µg/ml) were higher than ethyl acetate fraction (IC_{50} = 8.05 µg/ml) and ascorbic acid (IC_{50} = 17.17 µg/ml), respectively. Gallic acid exhibited strong antioxidant activities and this results were consistent with the report of Miliauskas, Venskutonis and Beek (2004). The high scavenging activity could be attributed to their free hydroxyl groups, and the phenolic hydroxyl structural group in benzene ring as depicted in Figure 2 (Shahidi, Janitha and Wanasundara, 1992).
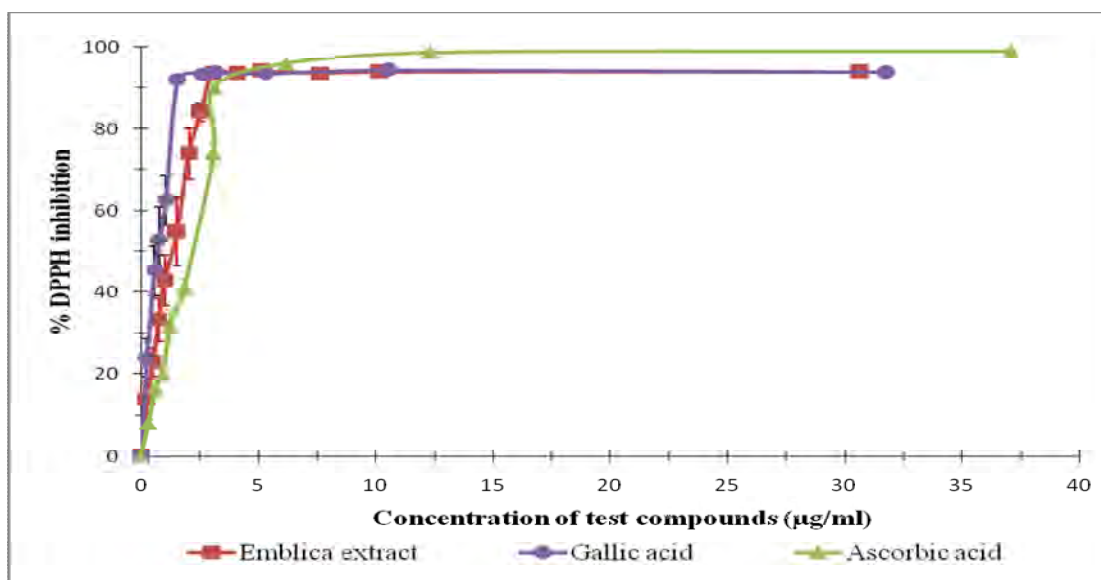
Figure 15. DPPH radical inhibition of the emblica extract compared to standard gallic acid and ascorbic acid (Mean ± SD, n=3)

Table 10 The IC_{50} values calculated from linear regression equations, showing R^2 for DPPH radical scavenging activity of each antioxidants

Sample	IC_{50} (µg/ml)	Mean (µg/ml)	SD	R^2
Emblica extract	1.52	1.38	0.14	0.9899
	1.38			0.9779
	1.25			0.9682
Gallic acid	0.85	0.74	0.10	0.9897
	0.65			0.9857
	0.73			0.9694
Ascorbic acid	2.08	2.09	0.06	0.9833
	2.04			0.9971
	2.15			0.9978
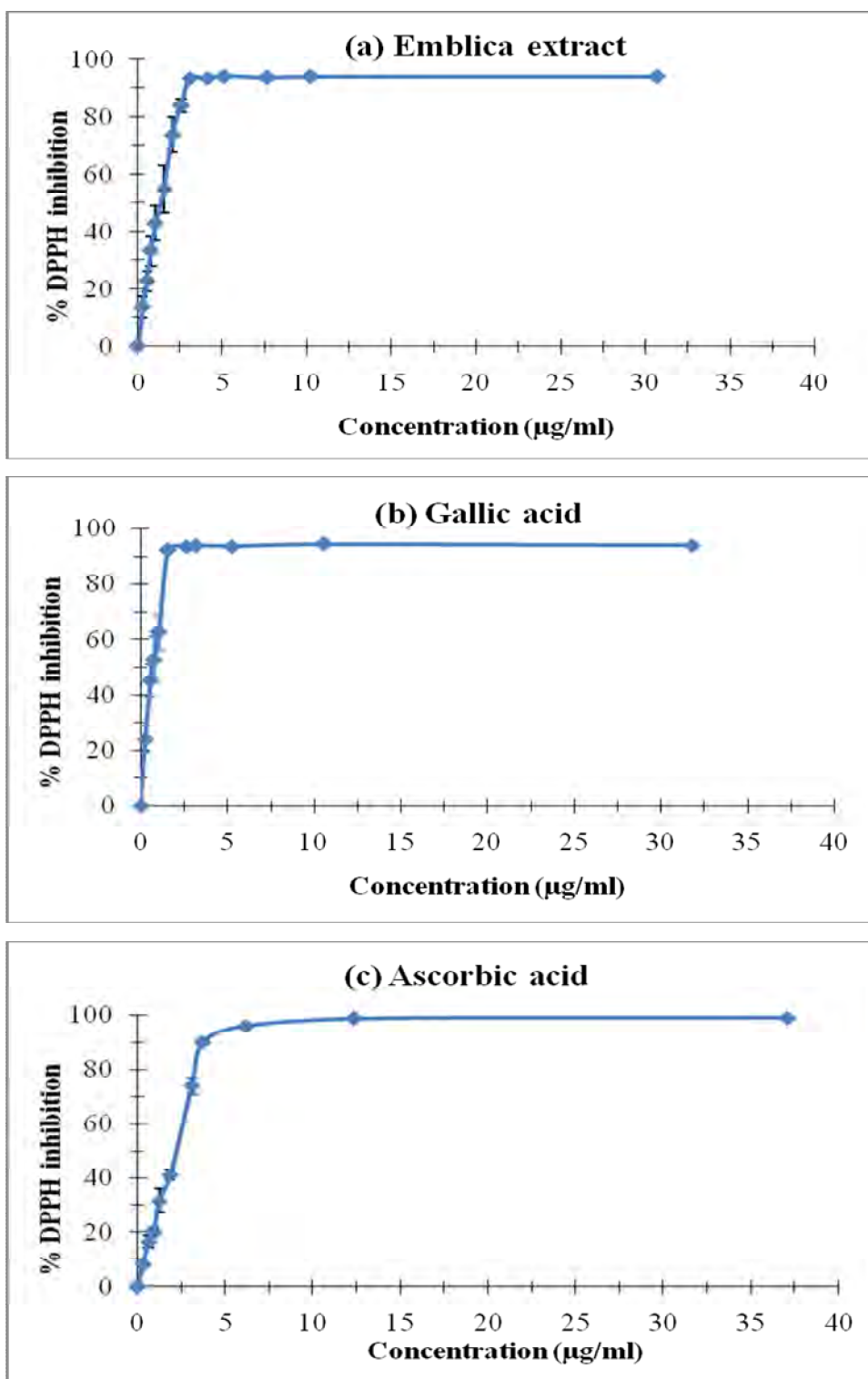
Figure 16. Relationship between percent DPPH radical inhibition and the concentration of the individual antioxidants (Mean ± SD, n =3) (a) emblica extract, (b) gallic acid and (c) ascorbic acid

D. Evaluation of Emblica Extract for Antityrosinase Activity

Tyrosinase is a copper-containing enzyme involved in producing high molecular weight brown pigments (melanins), which is the key physiological defense against sun-induced damages. Tyrosinase inhibitors have been increasingly important in medication and in cosmetic to prevent hyperpigmentation by inhibiting tyrosinase enzyme (Nerya et al., 2003).

The inhibitory effect on tyrosinase activity was examined by the dopachrome enzymatic method using L-DOPA as the substance (Nerya et al., 2004). Dopachrome was one of the intermediate substances in the melanin biosynthesis in which the red color could be detected by visible light. In the present study, the spectroscopic method using a microplate reader was used to measure the changes in absorbance at 492 nm due to formation of dopachrome. Kojic acid and alpha arbutin were used as a reference tyrosinase inhibitors in this study. The incubation time for antityrosinase activity of the test samples was set at 10 min according to the study of Jithavech (2005). A potent tyrosinase inhibitor would cause a decrease on dopachrome absorbance.

The percentage of anti-tyrosinase activity (% tyrosinase inhibition) of all test samples at various concentrations are shown in Figure 17. The relationship between inhibition and concentration of individual tyrosinase inhibitor is shown in Figure 18. The tyrosinase inhibiton activity of kojic acid exhibited the highest activity. Alpha arbutin showed the lowest activity as seen in Figure 17.

The concentration at 50% inhibition (IC_{50}) of each sample was determined by extrapolation of the plot between % inhibition and concentration and shown in Figures D1- D3 (Appendix D). The estimated IC_{50} values can be ranked from the lowest to the highest, as follows: kojic acid (0.0317 mg/ml ± 0.0029), gallic acid (0.8833 mg/ml ± 0.0760) and emblica extract (0.9708 mg/ml ± 0.0548). That result was in agreement with the publications of Nithitanakool et al. (2009) that kojic acid had higher activity than gallic acid. However, the IC_{50} of alpha arbutin was not able to be determined because its maximum activity was only at 24.15% inhibition. This might be attributed to the mechanism of antityrosinase activity of alpha arbutin. The mechanism involved that it competed with L-tyrosine for binding at the active site of tyrosinase. Alpha arbutin was a competitive inhibitor of tyrosinase when L-tyrosine is

the substrate. In contrast, L-dopa was used as an alternative substrate to L-tyrosine, alpha arbutin inhibited the oxidation of L-dopa to L-dopaquinone noncompetitively (Maeda and Fukuda, 1995).
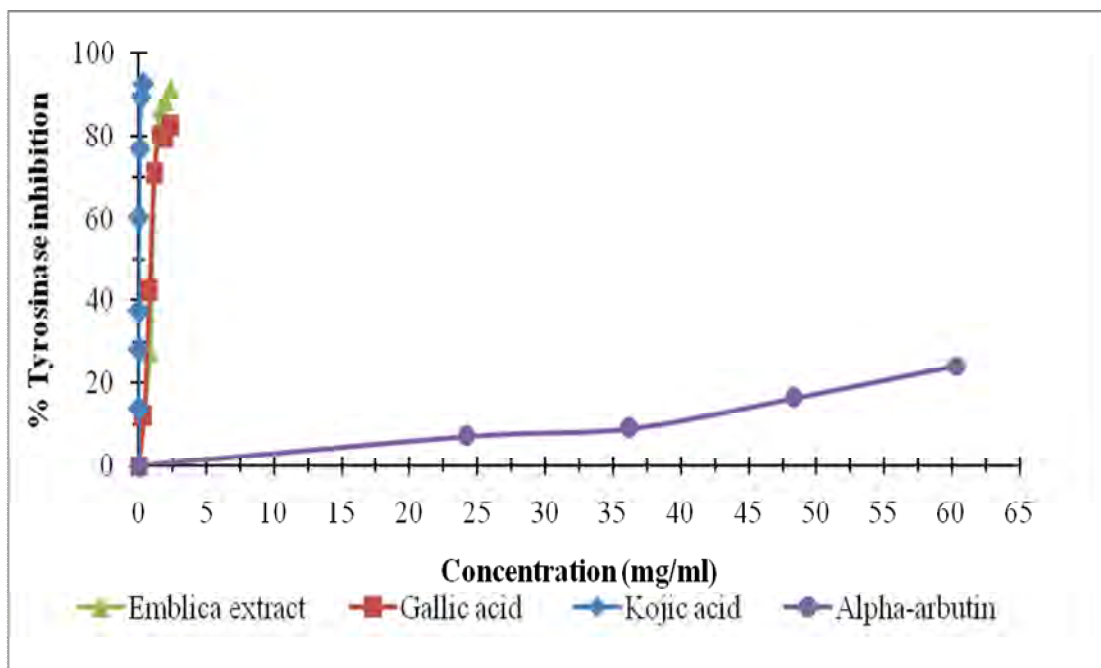
Figure 17 Tyrosinase inhibition of various emblica extract compared to standard gallic acid and positive controls (Mean ± SD, n=3)

The IC_{50} data were subsequently analyzed by one-way analysis of variance (ANOVA) at 95% confidence followed by Tukey method. The results are depicted in Tables D5 and D6 (Appendix D), respectively. It was found that there was a significant difference among the studies ($p < 0.05$).

According to Tukey method (Table D7 (Appendix D)), the antityrosinase activity could be ranked into 2 groups regarding their abilities to inhibit tyrosinase. The IC_{50} ranking is as follows:

Kojic acid > Emblica extract = Gallic acid

The IC_{50} value of gallic acid was similar to the previous reported by Kubo, Chen and Nihei (2003) that IC_{50} was 767 μg/ml. Kubo et al. (2003) reported that gallic acid reduced dopaquinone back to L-dopa by redox cycling in melanogenesis.

The IC_{50} value of emblica extract was similar to the report by Jithavech (2005) that shown IC_{50} of ethyl acetate extract was 1.19 mg/ml.

Table 11 The IC_{50} values of tyrosinase inhibition of each antityrosinases

Sample	IC_{50} value		
	IC_{50} (mg/ml)	Mean(mg/ml)	SD
Emblica extract	0.9075	0.9708	0.0548
	1.0025		
	1.0025		
Gallic acid	0.8000	0.8833	0.0760
	0.9000		
	0.9500		
Kojic acid	0.0300	0.0317	0.0029
	0.0350		
	0.0300		
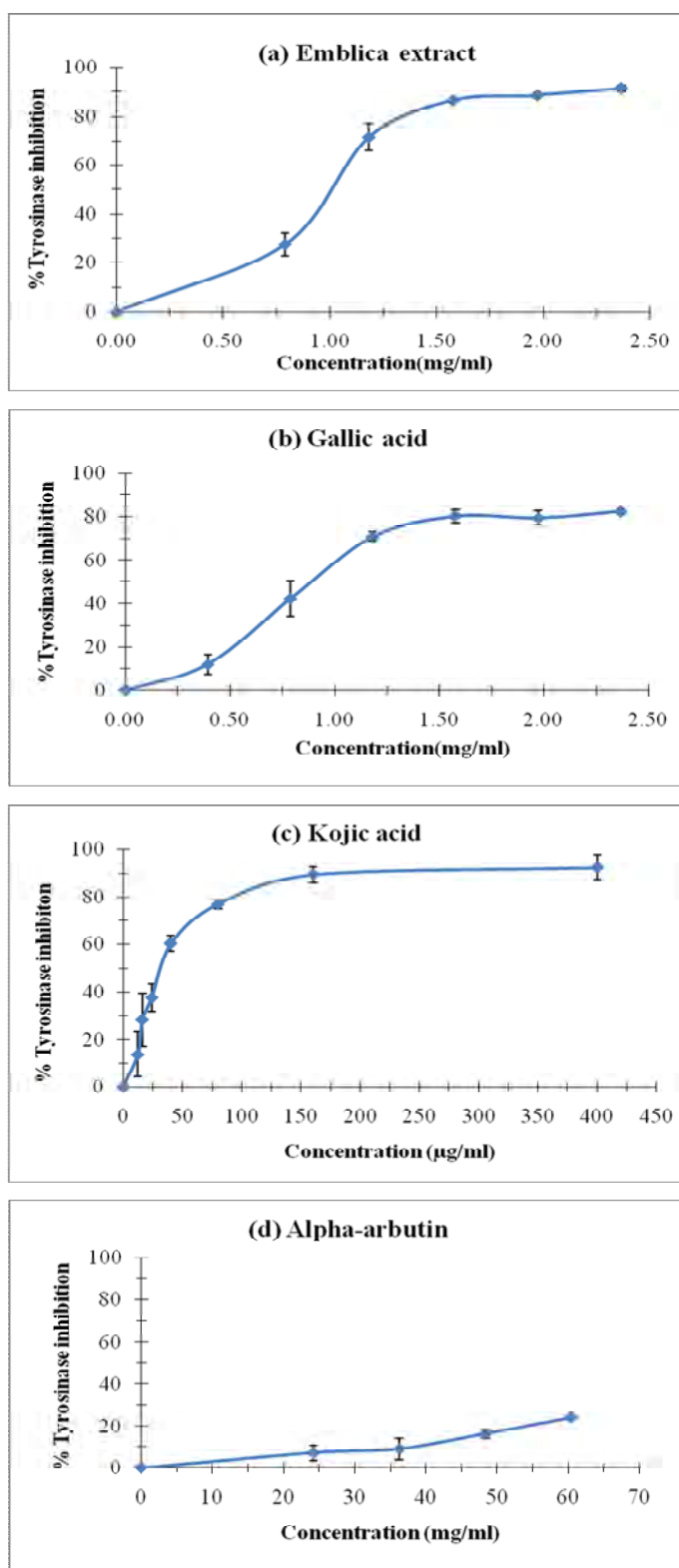
Figure 18 Relationship between percent tyrosinase inhibition and the concentration of the individual antityrosinases (Mean ± SD, n =3) (a) emblica extract, (b) gallic acid, (c) kojic acid and (d) alpha arbutin

E. Preparation and Characterization of Niosomes

1. Preparation of niosomes

There are different approaches for the production of niosomes. In this study, reverse phase evaporation technique was applied for the preparation of niosomes (Szoka and Papahadjopoulos,1978 ;   New, 1990 and Lasch et al., 2003). Conditions for forming w/o emulsion were the ratio of oil in solvent to aqueous phase (without and with 0.5% w/v emblica extract) at 3:1 and sonication time for 5 min in the ice bath sonicator. Evaporation conditions were 60 ºC, 450 mbar for 30 min. Then, w/o emulsion was evaporated and obtained gel attached to the round bottom flask. Gel collapsed would occur about 15 min after starting evaporation. Afterthat, reduced pressure evaporation was done to remove trace of solvent. Further size reduction was done by sonication for 60 min. The total lipid concentration was 60 µmol/ml aqueous phase. It was found that some niosome formulations could not be prepared. Span 40 and Span 60 at all surfactant:cholesterol ratios yielded niosomes, whereas Span 20 and Span 80 could gave niosomes at some surfactant:cholesterol ratios. The compositions of nine niosome formulations including blank niosome and with emblica extract which could be prepared are shown in Table 12. The formulation code was represented by N and four numbers. N means niosome, the first two figures 20, 40, 60 and 80 mean Span 20, Span 40, Span 60 and Span 80, respectively. The last two figures 11, 64, 73 mean surfactant:cholesterol ratio 1:1, 6:4 and 7:3, respectively.

Table 12. Compositions of the niosomes formulation including blank niosomes and with added 0.5% emblica extract (%w/v)

Code	Compositions	Ratio of surfactant : choleserol : Solulan C-24 (molar ratio)
N2011	Span 20 : Cholesterol :Solulan C-24	47.5 : 47.5 : 5
N2064	Span 20 : Cholesterol :Solulan C-24	57 : 38 : 5
N4011	Span 40 : Cholesterol :Solulan C-24	47.5 : 47.5 : 5
N4064	Span 40 : Cholesterol :Solulan C-24	57 : 38 : 5
N4073	Span 40 : Cholesterol :Solulan C-24	66.5 : 28.5 : 5
N6011	Span 60 : Cholesterol :Solulan C-24	47.5 : 47.5 : 5
N6064	Span 60 : Cholesterol :Solulan C-24	57 : 38 : 5
N6073	Span 60 : Cholesterol :Solulan C-24	66.5 : 28.5 : 5
N8064	Span 80 : Cholesterol :Solulan C-24	66.5 : 28.5 : 5

2. Characterization of physicochemical properties of emblica extract loaded niosomes

2.1. Physical appearance

Nine niosome preparations loaded with 0.5% emblica extract had light brown color as depicted in Figure 19. The color of the formulations was attributed to the color of emblica extract. All preparations appeared as milky or colloidal dispersion. The formulations other than mentioned could not result niosomes, which phase separation could be observed after addition of aqueous phase with or without 0.5% w/v emblica extract.
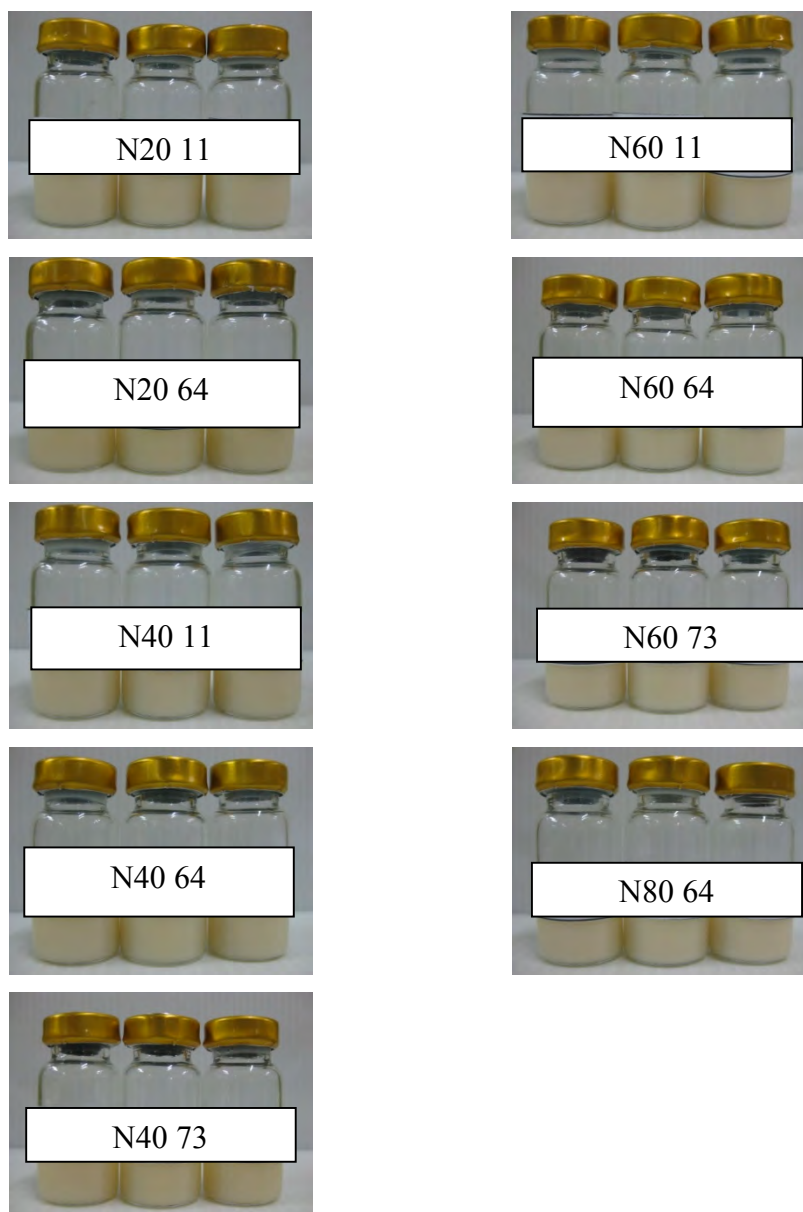
Figure 19 Photographs of niosome formulations with emblica extract

2.2. Microscopic appearances

The morphology of nine niosome formulations was determined by a Nikon Eclipse Model IX51inverted microscope. The photographs of emblica loaded niosomes formulations are shown in Figure 20.

All formulations exhibited spherical shape. From all figures shown, no aggregates particles was seen.
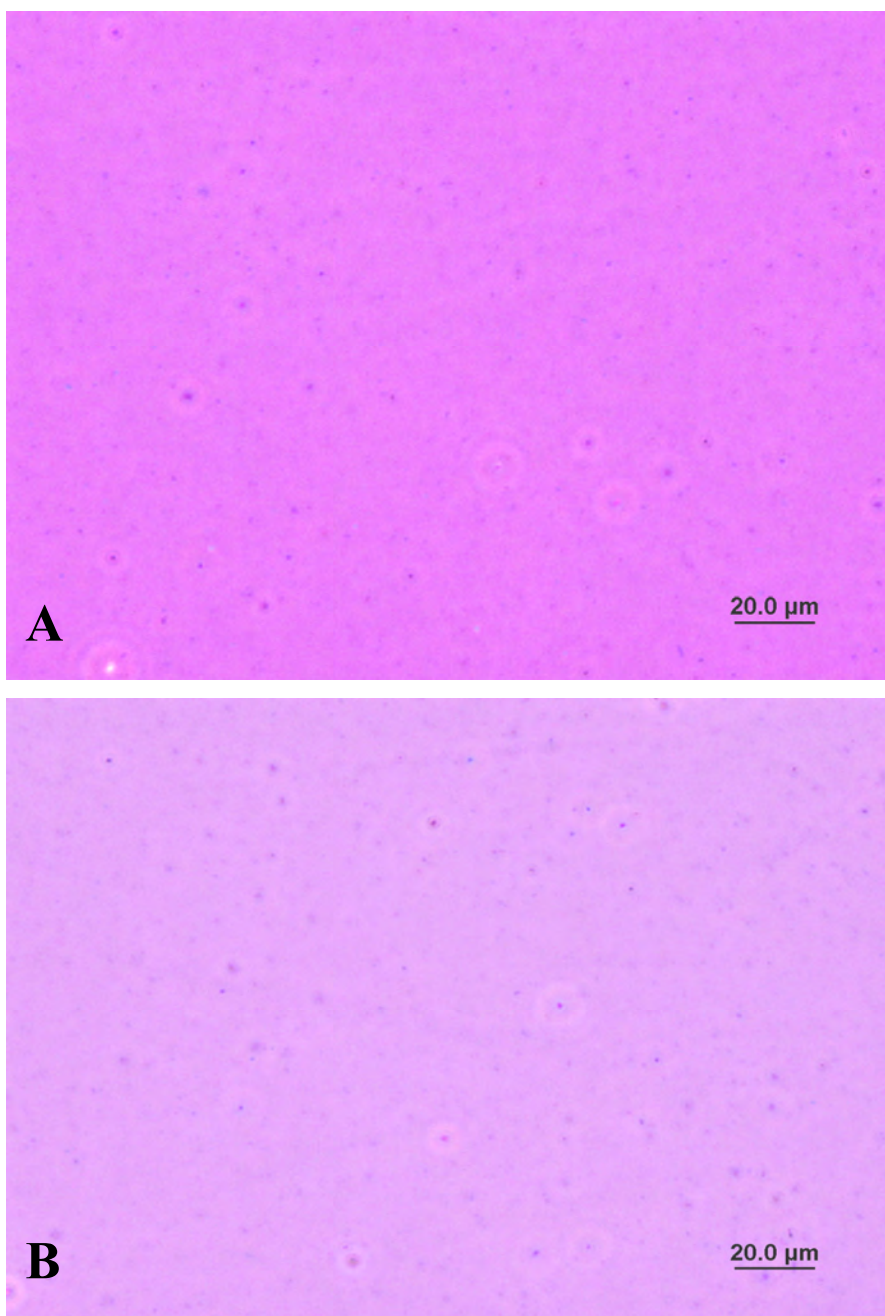
Figure 20 The photographs of freshly prepared niosomes loaded emblica extract with magnification 10x40 (A) N2011, (B) N2064, (C) N4011, (D) N4064, (E) N4073, (F) N6011, (G) N6064, (H) N6073, (I) N8064

Figure 20 The photographs of freshly prepared niosomes loaded emblica extract with magnification 10x40 (A) N2011, (B) N2064, (C) N4011, (D) N4064, (E) N4073, (F) N6011, (G) N6064, (H) N6073, (I) N8064 (Continued)

Figure 20 The photographs of freshly prepared niosomes loaded emblica extract with magnification 10x40 (A) N2011, (B) N2064, (C) N4011, (D) N4064, (E) N4073, (F) N6011, (G) N6064, (H) N6073, (I) N8064 (Continued)

Figure 20 The photographs of freshly prepared niosomes loaded emblica extract with magnification 10x40 (A) N2011, (B) N2064, (C) N4011, (D) N4064, (E) N4073, (F) N6011, (G) N6064, (H) N6073, (I) N8064 (Continued)

I
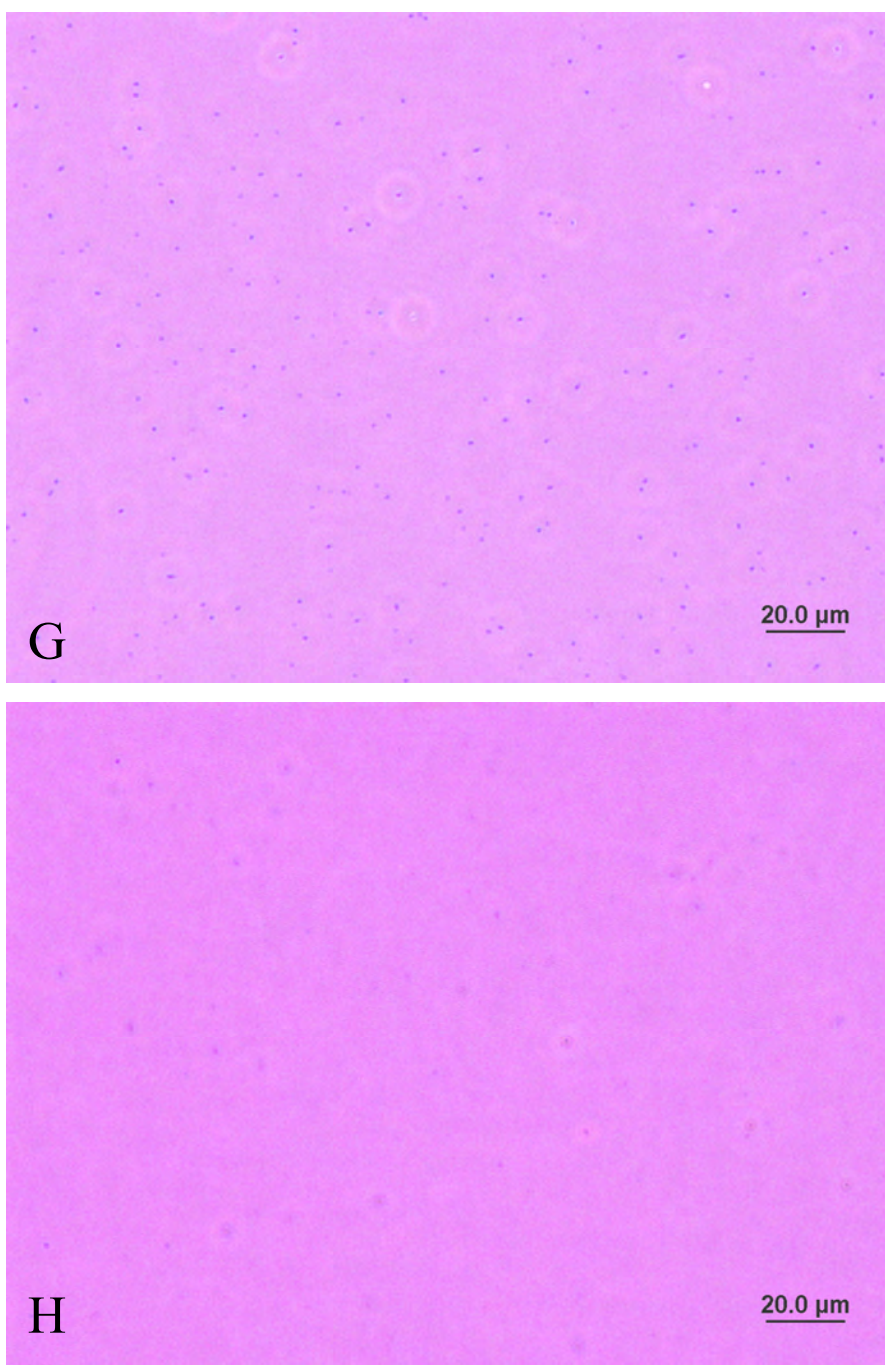
Figure 20 The photographs of freshly prepared niosomes loaded emblica extract with magnification 10x40 (A) N2011, (B) N2064, (C) N4011, (D) N4064, (E) N4073, (F) N6011, (G) N6064, (H) N6073, (I) N8064 (Continued)

     Cross polarized light microscopy have been used for detection of lamellar structure of bilayer membrane (Manosroi et al., 2003). This technique provides the observation of X-crosses of bilayer formation. Formulations in Table 12 could be observed X-crosses, for example as shown in Figure 21.
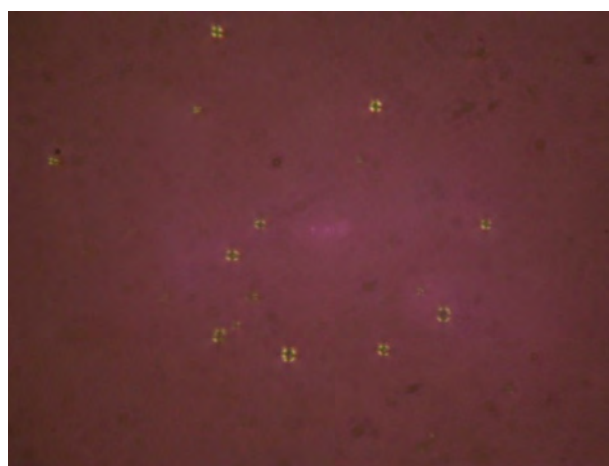
Figure 21 Cross polarized light microscopic appearance that shown X-cross of N2064 with magnification 10x100.

2.3. Particle size analysis

The particle sizes determined by PCS of blank niosome and emblica extract loaded niosomes using Span 20, Span 40, Span 60 and Span 80 as surfactant are shown in Table 13. The mean diameter at all preparations were in nanometer range as 142.1-259.0 and 215.7-323.0 nm. The size distribution as indicated by the polydispersity index were between 0.186-0.488 and 0.236-0.443. The particle size of all loaded niosomes with the emblica extract were larger than their corresponding blank niosomes. The aqueous solution of emblica extract was presumed to be entrapped in the core of the niosomal vesicles that resulted to the enlargement of the vesicular sizes. Moreover, at the pH of niosome formulation of approximately 3, a fraction of gallic acid entrapped (pKa = 4.40) might be in non dissociated form. The non dissociated gallic acid might adsorb and interact with the bilayer membrane and caused the enlargement of niosome.

Table 13 Particle size and polydispersity index of emblica extract loaded niosome formulations (mean±SD)

Formulation code	Blank formulation		Emblica extract loaded niosome formulation	
	Size (nm)	Pdi	Size(nm)	Pdi
N2011	259.4±5.63	0.187±0.010	306.2±17.6	0.267±0.061
N2064	242.6±8.97	0.345±0.052	273.0±7.9	0.333±0.061
N4011	163.9±1.43	0.225±0.007	245.8±5.0	0.373±0.087
N4064	142.1±0.98	0.186±0.004	215.7±17.0	0.236±0.040
N4073	183.2±0.49	0.230±0.004	323.0±8.2	0.333±0.093
N6011	208.5±0.99	0.244±0.005	240.9±12.6	0.383±0.133
N6064	173.2±0.96	0.223±0.005	216.2±21.6	0.371±0.100
N6073	177.3±0.31	0.267±0.002	234.7±20.9	0.256±0.010
N8064	182.9±3.69	0.488±0.060	301.6±39.6	0.443±0.014

It was noticeable that the polydispersity index of the formulation composed of Span80 (N8064) was higher than those of other formulations (blank niosome 0.488,

loaded niosome 0.443). This was corresponding to the size distribution curve as depicted in Figure 22. It showed the bimodal characteristic which revealed that there were diversity of particle sizes in the formulation.
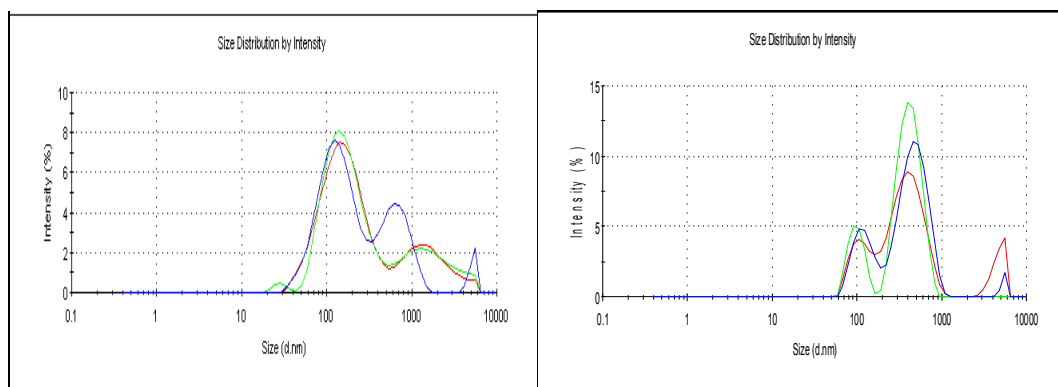
(a)   Blank formulation of N8064   (b) N8064 loaded emblica extract

Figure 22 Particle size analysis of N8064 formulation at initial time

2.3.1. Effect of type of surfactant

The effect of type of surfactant on particle size of niosomes was evaluated. As shown in Figure 23, the vesicle sizes of the formulations that composed of Span 20 were larger than those of the formulations that composed of Span 40 and Span 60. The ranking of sizes from varied types of surfactant might be as follows: Span 20 > Span 40 > Span 60. The vesicle size of the formulations might be attributable to the nature of alkyl chain of surfactant. An increasing the hydrophobicity of the surfactant monomer led to smaller vesicles, a result which might be anticipated since surface free energy decreased with increasing hydrophobicity (Yoshioka et al., 1994). The hydrophobic portions of Span 20 (monolaurate), Span 40 (monopalmitate) and Span 60 (monostearate) has C12, C16, C18 saturated alkyl chains, respectively. The longest saturated alkyl chain, C18; Span 60 led to smallest vesicles. The result of this study was similar to that reported by Yoshioka, Sternberg and Florence (1994) and Azeem et al (2008). They found that the vesicle sizes of loaded 5(6)-carboxyfluorescein and frusemide formulations composed of Span 20 (C12) larger than the formulations composed of Span 40 (C 16) and Span 60 (C18). It contributed that the mean size of niosomes increase proportionally with increase in the HLB of surfactants. As the surface free energy decreased with an increase in hydrophobicity of surfactant, size decreased.
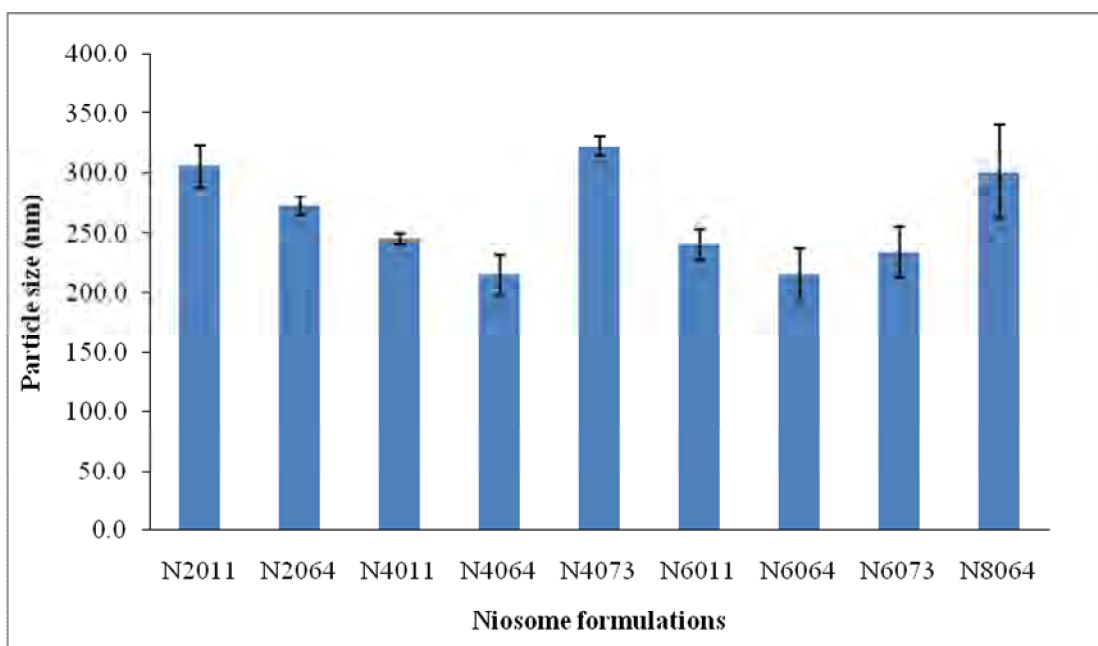
Figure 23 Histogram of particle size of emblica extract loaded niosome formulations, freshly prepared

2.3.2. Effect of cholesterol : surfactant ratio

The effect of surfactant:cholesterol ratio on the particle size of niosomes was evaluated. As shown in Figure 23, particle sizes of formulations that composed of surfacatant:cholesterol in 1:1 ratio were larger than those of the formulation that composed of surfactant :cholesterol in 6:4 ratio. But the formulation that composed of surfactant :cholesterol in 7:3 ratio were larger than the formulations that composed of surfactant:cholesterol in 6:4 ratio. The result was similar to Kapadia et al. (2009) that the vesicle sizes of the formulation that composed of Span 20 of surfactant:cholesterol ratio in 7:4, 7:6 and 1:1 were 3.31, 3.43 and 3.34 μm, respectively. This revealed that the effect of surfactant:cholesterol ratio on the particle size of niosomes was not clearly shown.

For physical stability study, particle size analysis of developed formulations was monitoring during 12 weeks at different storage temperatures, 4ºC and room temperature (RT). Particle sizes and dispersity indices of emblica extract loaded niosome formulations under storage conditions at 4ºC and RT during 12 weeks are illustrated in Table 14-15 and Figures 24-25.

Table 14 Particle size* and polydispersity index* of emblica extract loaded niosome formulations under storage condition at 4 ºC at initial, 2 weeks, 4 weeks, 8 weeks and 12 weeks

Code	Initial		Week 2		Week 4		Week 8		Week 12	
	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi
N2011	306.2 (17.6)	0.267 (0.061)	305.9 (23.5)	0.273 (0.066)	314.9 (28.4)	0.357 (0.052)	320.7 (29.3)	0.304 (0.101)	354.2 (30.1)	0.288 (0.060)
N2064	273.0 (7.9)	0.333 (0.061)	288.8 (25.1)	0.373 (0.129)	284.9 (28.3)	0.416 (0.046)	294.8 (26.1)	0.349 (0.106)	373.0 (39.7)	0.344 (0.087)
N4011	245.8 (5.0)	0.373 (0.087)	273.8 (36.0)	0.415 (0.050)	284.6 (20.3)	0.573 (0.051)	279.0 (41.1)	0.531 (0.066)	275.9 (35.6)	0.523 (0.094)
N4064	215.7 (17.0)	0.236 (0.040)	217.9 (30.3)	0.273 (0.054)	222.5 (32.7)	0.392 (0.085)	221.7 (24.1)	0.626 (0.047)	285.9 (14.4)	0.663 (0.071
N4073	323.0 (8.2)	0.333 (0.093)	328.0 (30.1)	0.343 (0.102)	335.8 (30.8)	0.399 (0.043)	337.0 (33.0)	0.339 (0.101)	386.9 (38.0)	0.358 (0.064)
N6011	240.9 (12.6)	0.383 (0.133)	243.1 (33.6)	0.390 (0.112)	243.8 (11.3)	0.431 (0.046)	261.3 (31.2)	0.417 (0.076)	345.5 (38.6)	0.433 (0.090)
N6064	216.2 (21.6)	0.371 (0.100)	232.9 (38.0)	0.370 (0.129)	235.9 (31.5)	0.483 (0.099)	256.8 (32.9)	0.390 (0.051)	300.1 (31.3)	0.504 (0.112)
N6073	234.7 (20.9)	0.256 (0.010)	235.1 (22.6)	0.254 (0.011)	237.8 (29.3)	0.363 (0.149)	248.4 (11.1)	0.312 (0.020)	291.4 (23.6)	0.340 (0.025)
N8064	301.6 (39.6)	0.443 (0.014)	Phase separation							

*Mean of nine measurements (SD), (n=3)

Table 15 Particle size* and polydispersity index* of emblica extract loaded niosome formulations under storage condition at room temperature (30 ºC) at initial, 2 weeks, 4 weeks, 8 weeks and 12 weeks

Code	Initial		Week 2		Week 4		Week 8		Week 12	
	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi	Size (nm)	Pdi
N2011	306.2 (17.6)	0.267 (0.061)	318.3 (24.0)	0.305 (0.075)	337.2 (28.9)	0.263 (0.051)	344.1 (23.8)	0.309 (0.071)	402.1 (24.6)	0.326 (0.070)
N2064	273.0 (7.9)	0.333 (0.061)	545.0 (50.1)	0.291 (0.080)	632.0 (27.7)	0.357 (0.123)	606.1 (73.4)	0.333 (0.079)	636.7 (78.2)	0.338 (0.060)
N4011	245.8 (5.0)	0.373 (0.087)	285.6 (41.1)	0.397 (0.099)	491.4 (47.2)	0.398 (0.077)	466.8 (41.5)	0.417 (0.107)	478.1 (38.6)	0.420 (0.100)
N4064	215.7 (17.0)	0.236 (0.040)	229.8 (17.9)	0.241 (0.039)	309.6 (16.6)	0.300 (0.068)	296.4 (17.9)	0.467 (0.066)	313.7 (17.5)	0.254 (0.058)
N4073	323.0 (8.2)	0.333 (0.093)	336.4 (24.7)	0.320 (0.009)	336.8 (24.9)	0.364 (0.071)	355.2 (19.7)	0.331 (0.068)	413.6 (26.3)	0.333 (0.070)
N6011	240.9 (12.6)	0.383 (0.133)	261.8 (42.7)	0.370 (0.107)	289.2 (46.5)	0.389 (0.142)	329.3 (46.0)	0.366 (0.115)	379.3 (52.1)	0.389 (0.120)
N6064	216.2 (21.6)	0.371 (0.100)	260.3 (49.2)	0.374 (0.171)	294.4 (64.6)	0.448 (0.113)	322.5 (48.1)	0.389 (0.174)	333.1 (57.3)	0.361 (0.102)
N6073	234.7 (20.9)	0.256 (0.010)	240.3 (23.5)	0.281 (0.023)	249.5 (25.4)	0.355 (0.016)	324.5 (21.7)	0.367 (0.134)	329.5 (25.5)	0.304 (0.010)
N8064	301.6 (39.6)	0.443 (0.014)	Phase separation							

*Mean of nine measurements (SD), (n=3)

There was phase separation observed from the niosome formulation using Span 80 after 2 weeks. This indicated that Span 80 could not give appropriate formulation at any surfactant:cholesterol ratios studied. From the particle size analysis of N8064 as shown in Figure 22. The size distribution curve showed the bimodal characteristic with large particle size with of 301.6±39.6 and high polydispersity index of 0.443±0.014. This meant that there were smaller vesicular size group and large vesicular size in the formulation. This resulted finally in aggregation and phase separation under storage at both conditions. Additionally, this might be also involved with structure of Span80 which composed of unsaturated bond in alkyl chain. This

meant that the adjacent molecular structure could not be tightly arranged when they formed the membrane of niosomes (Hao et al., 2002).

At storage temperature at 4 °C, the results showed that the vesicle sizes of nearly all formulations had no appreciable change (Figure 24). This could be due to decreased mobility of the bilayer at lower temperature, thus offering no change of particle size, whrereas at room temperature the particle size was increased which might be due to the fusion of smaller vesicles to form larger ones (Balasubramaniam, Kumar and Pillai, 2002). It might be seen that nearly all niosome formulations were rather physically stable at refrigerator temperatures during the period of study. In contrast, under storage at room temperature, there were a remarkable increase of vesicle sizes of some formulations, for example, niosomes formulations using Span 20 (N2064) after 2 weeks, and Span 40 (N4011) after 4 weeks.

From the results of both storage conditions, it seemed that niosome formulations which exhibited noticeable physical stability as compared with other formulations, N4064 and N6064.
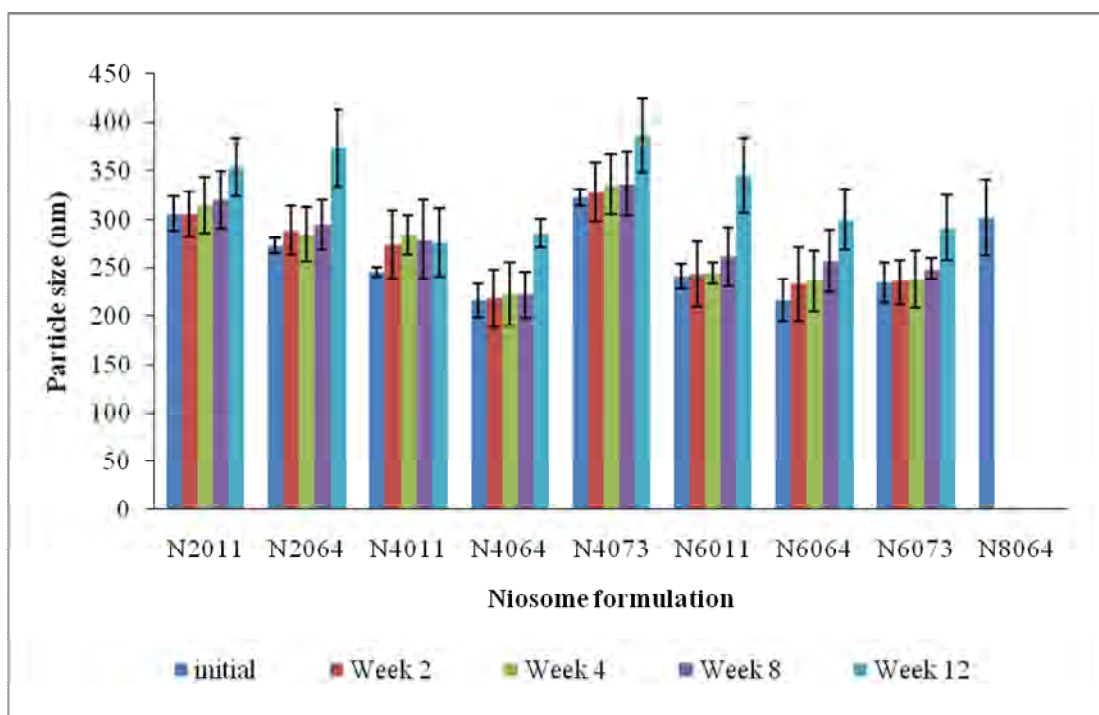
Figure 24 Plots of particle size of emblica extract loaded niosome formulations under storage at 4° C during 12 weeks (not available data in some observations due to phase separation)
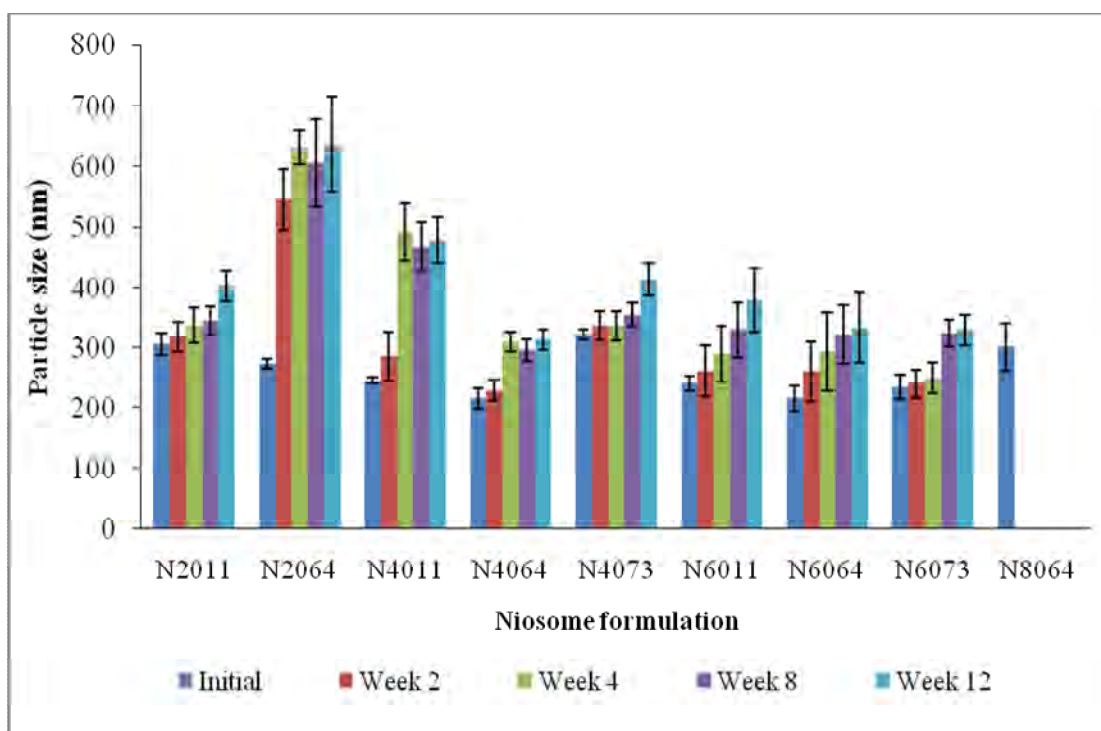
Figure 25 Plots of particle size of emblica extract loaded niosomes formulation under storage at room temperature (30º C) during 12 week (not available data in some observations due to phase separation)

2.4 Entrapment efficiency

The determination of gallic acid in emblica extract loaded niosomes was carried out by HPLC because of its specificity and high sensitivity.

The validation of analytical method is the process which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. The analytical parameters practically used for the assay validation were specificity, linearity, accuracy and precision. The data was demonstrated in the Appendix B1.

The determination of amount of entrapped gallic acid in niosomes dispersion was carried out by using centrifugation method. The entrapment efficiency study of gallic acid loaded niosomes was observed during storage time of 12 weeks, (initial time, week 2, week 4, week 8, week 12).

2.4.1. Effect of type of surfactant

Type of surfactants influences encapsulation, toxicity and stability of niosomes. Span surfactants with HLB values between 4 and 8 were found to be compatible with vesicle formation (Uchegbu and Vyas, 1998).

The effect of type of surfactant on entrapment efficiency was evaluated. The entrapment efficiency of all formulations are shown in Table 16 and Figure 26. At any surfactant to cholesterol ratios, the formulation that composed of Span 20 had entrapment efficiency higher than the formulations that composed of Span 40 and Span 60.

One possible explanation to the result might involve the vesicular size of niosomes. Span 20 (N2011 and N2064) gave vesicular sizes which were mostly larger than those of Span 40 and Span 60. This might be attributed to the higher entrapment efficiency of Span 20.

By chemical structure, all Span types have the same hydrophilic head group but alkyl chain in different lengths. Span 20, Span 40, Span 60 are monolaurate (C12), monopalmitate (C16) and monostearate (C18), respectively, whereas Span 80 is monooleate (C18) with an unsaturated double bonds. Hao and Li (2011) reported that increase of the alkyl chain length led to a higher entrapment efficiency, and the corresponding order follows the trend Span 60 (C16) > Span 40 (C14) which the length of alkyl chain is a crucial factor that should be considered in niosomes system with high efficiency, whereas, Span 60 and Span 80 have the same head groups and same length of alkyl chain but Span 80 has an unsaturated double bonds that caused a marked explaining the lower entrapment than Span 60.

Table 16 Entrapment efficiency of emblica extract loaded in niosomes

Formulation code	% Entrapment efficiency
N2011	39.28±4.11
N2064	49.29±4.60
N4011	38.20±1.49
N4064	36.97±1.75
N4073	34.35±1.81
N6011	38.27±1.25
N6064	41.45±2.32
N6073	36.23±1.93
N8064	41.26±2.17
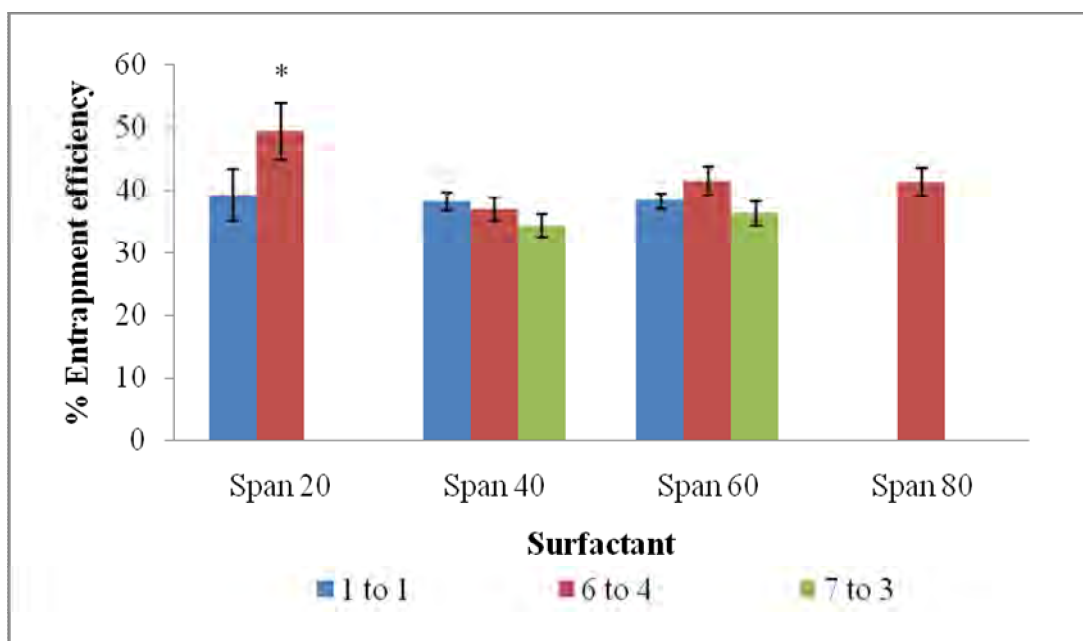
Figure 26 Percentage entrapment efficiency of niosomes formulation (mean±SD; n=3)
* = significant different from other formulation (p<0.05, ANOVA)

2.4.2. Effect of surfactant:cholesterol ratio

From the results, surfactant:cholesterol ratio had some noticeable effect on entrapment efficiency of the formulation studied. For most surfactants studied, the ratio of 6:4 showed the superior entrapment efficiency to the ratio of 1:1 and 7:3.

This revealed that the concentration of surfactant as relative to cholesterol concentration should be at an optimal level, which depended on the type of surfactant.

At ratio of surfactant to cholesterol 6 to 4, the entrapment efficiency of the formulations of N2064, N4064 and N6064 were 49.29, 36.97 and 41.45%, respectively. These results revealed that type of surfactant affected loading capacity of niosomes.

After statistical analysis with one-way ANOVA and Tukey test at 5% significant level, there were significant differences between varied compositions of niosomes dispersion as depicted in Table E20 and E21 (Appendix E). The N2064 formulation had the highest entrapment efficiency. This formulation was composed of Span 20 with the Span 20:cholesterol ratio of 6:4. This result was similar to Shahiwala and Misra (2002) that entrapment efficiency of nimesulide that composed of Span 20 had higher entrapment efficiency than the formulation that composed of Span 60. The possible explanation that the N2064 formulation had the highest entrapment efficiency might be due to the appropriate of surfactant type and surfactant:cholesterol ratio (at 6:4). Span 20 has the shortest alkyl chain which could decrease less surface free energy than other surfactant Span 40, Span 60, Span 80. This might be a possible reason that its niosome entrapped the emblica extract with high efficiency.

For leakage study of entrapped emblica extract in all niosome formulations, the percentages entrapment efficiency were monitored at different times for 12 weeks under storage temperature at 4 ℃ and room temperature (30 ℃). The entrapment efficiency data are shown in Table 17 and Table 18.

At both temperatures, the formulation with Span 80 (N8064) failed in the leakage study due to phase separation was observed at the second week.

The percentage entrapment efficiency of all formulations decreased with an increasing of storage duration. The decrease of entrapment efficiency were ranged from 4.00-7.65 at 4 ℃, whereas from 6.02-9.70 at room temperature. The effect of storage temperature on the decrease of entrapment efficiency could be detected, but in small values. This might be correspond to the change of particle size of niosomes at storage temperature at 4 ℃ and room temperature. That larger particle sizes were resulted at higher temperature (Table 15 and Figure 25). The leakage of

entrapped emblica extract might be due to the degradation of the niosomal bilayers. The degradation of surfactants in the bilayers resulting in defects in membrane packing making them leak (Agrawal, Katare and Vyas, 2001). All of formulation composed of sorbitan ester group which had the hydrolysis reaction of alkyl esters, especially at pH below 4 (Rieger, 1997).

After statistic analysis with ANOVA ($\alpha=0.05$) and Tukey method. There were significant differences between time for storage condition as depicted in Tables 17 and 18. At 4ºC storage condition for 3 months, the formulation composed of Span20 (N2011) showed a significant decrease of entrapment efficiency ($p<0.05$) from the initial time, whereas at room temperature, both formulations of Span20 (N2011 and N2064) exhibited significant decreases of entrapment efficiency ($p<0.05$). This result was similar to Yoshioka, Sternberg and Florence (1994) that the leakage of 5(6)-carboxyfluorescein loaded Span surfactant niosomes of Span 20 more than the formulation that composed of Span 40 and Span 60.

The leakage of the formulations might be due to phase transition temperature of surfactant. The phase transition temperature of the vesicle systems indicated the thermodynamic state of the bilayers. Molecules of Span 20 (Tc = 16ºC) are in the liquid crystalline state, whereas molecules of Span 60 (Tc = 53ºC) are in the ordered gel state, which the hydrocarbon chain are fully extended and closely packed (Suwakul, Ongpipattanakul and Vardhanabhuti., 2006). The higher phase transition temperature of surfactant appeared to result in more stability of vesicles. Therefore, the formulations that composed of Span 60 had more stability than the formulations that composed of Span 20.

Bilayer composition with addition of different additives along with surfactants could improve the stability of niosomes. Inclusion of cholesterol increased niosomal hydrodynamic diameter and entrapment efficiency. An increase in cholesterol content of bilayers resulted an increase of the rigidity, reduction of leakage and improvement of stability of the bilayer (Agrawal et al., 2001).

The effect of surfactant:cholesterol ratio to the change of entrapment could be seen from Table 17 and Table 18. At the ratio of 6:4 (lower cholesterol content) the change seen a little higher than that at the ratio of 5:5 (higher cholesterol content). Addition of Solulan C-24 (cholesteryl poly-24-oxyethylene ether) was

reported to use for prevention of aggregation due to development of steric hindrance in the colloidal system. Usually, it was added in small molar ratio with cholesterol (Junyaprasert, Teeranachaideekul, and Supaperm, 2008). In this study, the fixed molar ratio of Solulan C-24 was used.

Based on the result of this study, the formulation with surfactant:cholesterol ratio of 6:4 gave high entrapment efficiency whereas, the formulation N2064 gave the highest entrapment efficiency of 49.29%. Then, it was rational for selection this ratio of surfactant: cholesterol (N2064, N4064 and N6064) for further serum formulations.

Table 17 Entrapment efficiency of emblica extract loaded in niosomes at 4ºC(value expresses as mean±SD, n=3)

Formulation	% Entrapment efficiency					Change
	Initial time	Week 2	Week 4	Week 8	Week 12	
N 20 11	39.28±4.11	37.52±4.00	36.02±3.72	34.57±2.70	32.72±3.42*	6.56
N 20 64	49.29±4.60	43.66±4.52	44.28±4.54	43.07±3.75	41.64±2.35	7.65
N 40 11	38.20±1.49	35.31±1.85	33.66±1.87	33.05±2.01	31.59±2.56	6.61
N 40 64	36.97±1.75	34.58±1.72	35.90±1.18	34.90±1.07	30.15±3.64	6.82
N 40 73	34.35±1.81	30.47±1.65	32.57±0.51	31.86±0.29	28.89±4.14	5.46
N 60 11	38.27±1.25	37.58±1.64	35.22±2.36	33.07±2.92	32.91±2.63	5.36
N 60 64	41.45±2.32	37.59±1.57	38.10±1.61	36.75±1.22	34.42±4.97	7.03
N 60 73	36.23±1.93	37.10±1.84	33.54±2.97	33.19±2.54	32.23±2.25	4.00
N 80 64	41.26±2.17	phase separation				

* = significant different from initial entrapment efficiency ($p < 0.05$, ANOVA)

Table 18 Entrapment efficiency of emblica extract loaded in niosomes at room temperature (30 ºC) (value expresses as mean±SD, n=3)

Formulation	% Entrapment efficiency					Change
	Initial time	Week 2	Week 4	Week 8	Week 12	
N 20 11	39.28±4.11	38.20±2.73	35.89±5.27	34.92±2.73	33.26±2.56*	6.02
N 20 64	49.29±4.60	47.62±5.93	45.00±4.91	43.88±5.29	40.65±1.42*	8.64
N 40 11	38.20±1.49	35.99±4.81	34.63±3.37	33.72±5.20	30.26±3.93	7.94
N 40 64	36.97±1.75	37.32±0.96	36.07±2.13	35.20±1.76	30.40±2.44	6.57
N 40 73	34.35±1.81	32.90±1.59	31.65±1.13	31.37±0.94	27.98±3.15	6.37
N 60 11	38.27±1.25	37.41±1.72	34.14±1.93	31.77±1.97	31.05±1.35	7.22
N 60 64	41.45±2.32	38.95±0.98	36.79±1.62	35.23±2.39	31.75±5.04	9.70
N 60 73	36.23±1.93	33.98±2.72	32.48±1.93	30.12±1.82	30.06±2.20	6.17
N 80 64	41.26±2.17	phase separation				-

* = significant different from initial entrapment efficiency (p<0.05, ANOVA)

2.5 Chemical stability of emblica extract loaded in niosomes

The percentage gallic acid remaining at storage condition at 4 ºC and room temperature (30 ºC) are shown in Table 19 and 20. At both storage conditions, percentage gallic acid content decreased with time. It might be due to oxidative degradation of gallic acid by oxygen to quinone compound (Pospisil, Cvikrova and Hruboov,1983; Melo, Leal, and Botelho, 2010).

The losses of percentage content of gallic acid in niosome formulations were exhibited as 5.83-9.26% at 4ºC, and as 8.95-20.05% at room temperature. At higher temperature, the degradation of gallic acid was much higher than at 4 ºC. It might be due to thermal degradation of decarboxylation of gallic acid to pyrogallol compound (Pajares et al., 2010). At the refrigerated temperature for 3 months, the loss was between 5.83-9.26%, which was less than 10% that it was acceptable for niosomal formulation (ICH Q1A). After statistic analysis with ANOVA, there was no significant difference of % loss between niosome formulations.

From the stability data of niosomes at room temperature, most formulations had the loss of gallic acid content between 8.95-20.05% which were more than 10% loss. There was no significant difference of % loss between niosome

formulations (ANOVA, p>0.05). This result suggested that the appropriate storage condition for niosomes might be refrigerated temperatures and protected from light.

Table 19 Percentage content of emblica extract loaded in niosome at 4 ℃ (value expresses as mean±SD, n=3)

Formulation	Content of gallic acid					
	Initial time	Week 2	Week 4	Week 8	Week 12	% Loss
N 20 11	100	97.79±0.97	96.83±0.37	97.31±0.96	90.74±2.13	9.26
N 20 64	100	98.67±1.16	96.45±0.78	93.02±1.30	92.45±3.54	7.55
N 40 11	100	99.95±0.99	99.88±1.82	94.36±4.07	92.34±2.98	7.66
N 40 64	100	98.69±0.49	98.03±1.56	96.21±0.72	92.80±4.86	7.20
N 40 73	100	95.90±1.52	95.27±2.43	93.79±1.10	92.80±1.83	7.20
N 60 11	100	99.60±1.85	97.62±1.66	95.87±2.17	91.17±2.38	8.83
N 60 64	100	98.52±0.88	96.99±0.76	96.26±0.78	93.87±1.68	6.13
N 60 73	100	98.29±1.59	98.08±1.25	96.36±0.46	94.17±1.39	5.83
N 80 64	100	phase separation				-

Table 20 Percentage content of emblica extract loaded in niosome at room temperature (value expresses as mean±SD, n=3)

Formulation	Content of gallic acid					
	Initial time	Week 2	Week 4	Week 8	Week 12	% Loss
N 20 11	100	95.77±1.53	93.36±2.85	91.75±3.12	79.95±5.39	20.05
N 20 64	100	98.34±2.71	95.07±3.36	92.95±6.72	81.98±6.34	18.02
N 40 11	100	98.87±6.22	93.43±6.99	90.73±5.45	88.79±6.80	11.21
N 40 64	100	98.46±1.55	97.57±3.01	96.58±1.06	91.05±3.72	8.95
N 40 73	100	96.79±2.31	94.47±4.40	88.42±3.09	85.48±2.97	14.52
N 60 11	100	96.68±5.82	94.12±4.75	91.15±8.09	84.00±7.25	16.00
N 60 64	100	97.39±1.61	95.26±4.56	91.44±5.88	84.13±6.78	15.87
N 60 73	100	97.01±3.44	97.50±0.58	92.10±1.78	85.85±9.65	14.15
N 80 64	100	phase separation				-

F. Preparation of Niosome Serums

   1. Formulation of niosome serum

Poloxamer 407 was selected for viscosity enhancement due to its nonionic property and the easiness in preparation. From the preliminary study, varied concentrations of Poloxamer 407 were studied.

It was found that the most appropriate concentration was 32% w/v, which after dilution with 1:1 ratio with niosomal dispersion, the concentration would become 16% w/v. The viscous dispersion of niosomes as called "serum" was obtained. Serum defined as a dispersion system that should be as viscous liquid, which practically should be pumped from the container.

   2. Characterization of niosome serums

The optimum formulations of niosomes which were selected from the above study, were N2064, N4064 and N6064. They were mixed with Poloxamer 407 with 1:1 mixing ratio. The viscous dispersion system (serum) coded as NS2064, NS4064 and NS6064.

The present study evaluated appearance, pH, viscosity and chemical stability of serums.

   2.1 Physical appearance

The serums had light brown color due to color of niosomal dispersion. The physical appearance of serum formulations were visually examined and as depicted in Figure 27. All formulations of serum that kept at 4 ºC showed separation of niosomal dispersion from poloxamer dispersion. This result might be occurred from the thermorevesible property of Poloxamers. Poloxamer 407 had characteristic of reverse thermal gelation. It was liquid at refrigerated temperature (4-5ºC), but became gel upon warming to room temperature (Escobar-Chavez et al., 2006). The separation of niosomes from serum that kept at 4 ºC might be due to density the difference between two systems (Figure 28). However, the separated serum could be reversibly disperse upon shaking the serum. As expected the separation of niosome serum that kept at room temperature (30ºC) was not occurred (Figure29).
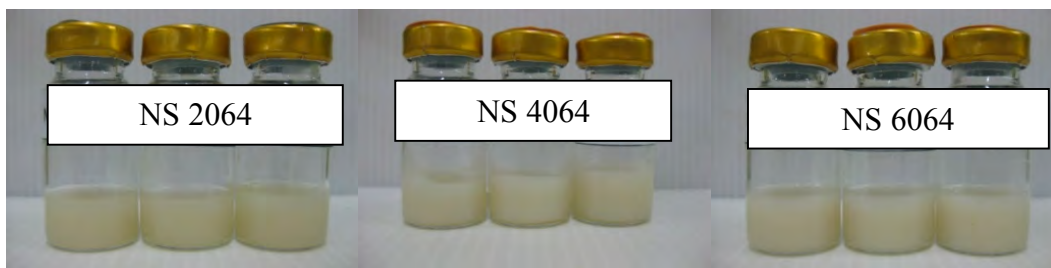
Figure 27 The photograph of niosome serum formulations with 0.5% emblica extract loaded niosomes, freshly prepared
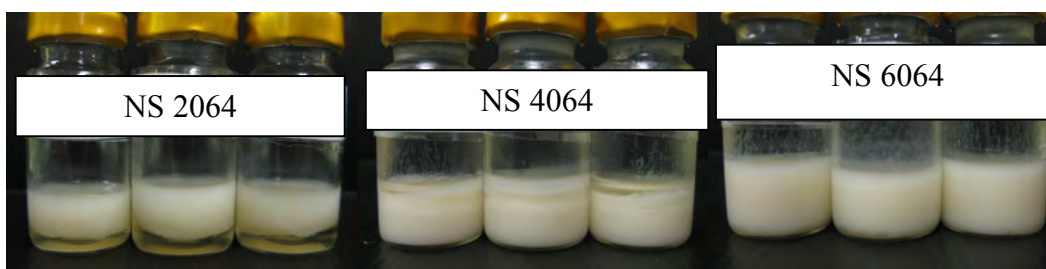
Figure 28 The photograph of niosome serum formulations with 0.5% emblica extract loaded niosomes, under storage condition at 4 ºC at 2 weeks

Figure 29 The photograph of niosome serum formulations with 0.5% emblica extract loaded niosomes, under storage condition at room temperature (30 ºC) at 12 weeks

2.2 pH

The pH values of serum formulations are shown in Table 21-22. The pH values of the systems were ranged between 3.54-3.79 at the initial time depending on the entrapment efficiency of gallic acid in niosomes. The pH values were ranged between 3.52-3.79 at 4 ºC and 3.51-3.83 after 12 weeks. There were no statistic significant difference (ANOVA) of pH from initial time with 2 weeks, 4 weeks, 8 weeks and 12 weeks, at $p>0.05$. This meant that there was no pH change in the study. Additionally, the pH of the serum formulation which was determined as pH 4, was dermatologically acceptable.

2.3 Viscosity

The viscosities of serum formulations are shown in Tables 21 and 22 . The viscosity values were ranged between 779-1154 centipoises at the initial time. There was no significantly difference among 3 formulations of serum. And the viscosity values were observed between 1003-1210 centipoises at 4 ºC and between and between 779-1372 centipoise at room temperature. It was found that the values were not significantly different from those at the initial time ($p > 0.5$). Statistical analysis with ANOVA at 5% significant level of the viscosity of all NS formulations are depicted in Table F13-F24 (Appendix F).

2.4 Chemical stability

Chemical stability of serums were performed at both 4 ºC and at room temperature condition (30ºC) for 12 weeks in tight containers and protected from light. The determination of gallic acid content in niosome serum was performed by HPLC method. The analytical parameters used for the assay validation were specificity, linearity, accuracy and precision. The data was demonstrated in the Appendix B1.

The percentages remaining of gallic acid in serum are shown in Tables 23-24. At refrigerated temperature, the degradation of gallic acid was between 17.55-22.72%. After statistic analysis with ANOVA, NS4064 and NS6064 had a significant decrease of gallic acid content from initial time ($p < 0.05$). At room temperature, the degradation of gallic acid was between 28.85-35.91%. Similarly, only NS4064 had significant decrease of gallic acid content from initial time (ANOVA, $p < 0.5$). The degradation of gallic acid in niosome serum formulations was involved with poloxamer 407 that using as viscosity enhancer. Poloxamer 407 is a block copolymer that consist of ethylene oxide (EO) and propylene oxide (PO) in a structure with surfactant property with HLB of 22 (Dumortier et al., 2006). It might be due to surface active property of poloxamer 407 that brought some interfering effect to the bilayer and formation of membrane. The leakage of gallic acid niosomal vesicles to the external medium after week 8, that the degradation of free gallic acid was accelerated. The degradation of gallic acid which was a polyphenolic compound, was occurred by oxidation by atmospheric oxygen to quinone compound (Pospisil,

Cvikrova and Hruboov 1983; Melo, Leal and Botelho, 2010) and thermal degradation to pyrogallol compound (Pajares et al., 2010).

Table 21 Physicochemical characterizations of niosome serum formulations at 4 ℃

Formulation	Initial time		Week 2		Week 4		Week 8		Week 12	
	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH
NS2064										
lot1	1137	3.59	1016	3.59	1013	3.57	1034	3.59	1112	3.62
lot2	1041	3.59	1112	3.54	1056	3.59	1043	3.57	1003	3.60
lot3	1154	3.54	1125	3.58	1023	3.52	1117	3.58	1189	3.52
Mean±SD	1111±61	3.57±0.03	1084±60	3.57±0.03	1031±23	3.56±0.04	1065±46	3.58±0.01	1101±93	3.58±0.05
NS4064										
lot1	890	3.68	1002	3.69	1015	3.57	987	3.63	1041	3.70
lot2	1115	3.68	979	3.64	989	3.59	981	3.62	1067	3.68
lot3	779	3.59	1023	3.58	987	3.58	884	3.72	1031	3.67
Mean±SD	928±171	3.65±0.05	1001±22	3.64±0.06	997±16	3.58±0.01	951±58	3.66±0.06	1046±19	3.68±0.02
NS6064										
lot1	1113	3.79	1134	3.65	982	3.63	1158	3.75	1103	3.78
lot2	993	3.59	1013	3.67	942	3.71	987	3.79	1024	3.74
lot3	1096	3.68	1210	3.67	870	3.70	1121	3.72	1270	3.77
Mean±SD	1067±65	3.69±0.1	1119±99	3.66±0.01	931±57	3.68±0.04	1089±90	3.75±0.04	1132±125	3.76±0.02

Table 22 Physicochemical characterizations of niosome serum formulations at room temperature.

Formulation	Initial time		Week 2		Week 4		Week 8		Week 12	
	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH	viscosity (cps)	pH
NS2064										
lot1	1137	3.59	1106	3.52	1113	3.58	1148	3.61	1165	3.66
lot2	1041	3.59	1094	3.51	1182	3.58	1152	3.60	1163	3.66
lot3	1154	3.54	1165	3.59	1037	3.45	1124	3.62	1030	3.53
Mean±SD	1111±61	3.57±0.03	1122±38	3.54±0.04	1111±73	3.54±0.08	1141±15	3.61±0.01	1119±77	3.62±0.08
NS4064										
lot1	890	3.68	1012	3.68	1069	3.54	837	3.73	1014	3.72
lot2	1115	3.68	958	3.68	1049	3.58	842	3.72	1367	3.73
lot3	779	3.59	1179	3.65	844	3.63	904	3.74	1140	3.69
Mean±SD	928±171	3.65±0.05	1050±115	3.67±0.02	987±125	3.58±0.05	861±37	3.73±0.01	1174±179	3.71±0.02
NS6064										
lot1	1113	3.79	1374	3.63	844	3.64	1256	3.78	1113	3.83
lot2	993	3.59	1027	3.64	933	3.73	964	3.75	1241	3.76
lot3	1096	3.68	1316	3.68	887	3.74	1136	3.75	1372	3.75
Mean±SD	1067±65	3.69±0.1	1239±186	3.65±0.03	888±45	3.70±0.06	1119±147	3.76±0.02	1242±30	3.78±0.04

Table 23 Percentage contents of gallic acid remaining in serum during 12 weeks under storage condition at 4 ºC (mean±SD, n=3)

Formulation	Gallic acid content (%)					% Loss
	Initial	Week2	Week4	Week8	Week12	
NS2064	100	97.72± 1.21	92.26±1.95	91.90± 1.36	82.45± 5.92	17.55
NS4064	100	97.41± 1.71	90.34±4.77	84.96± 3.82	77.28±1.96*	22.72
NS6064	100	91.45± 6.98	89.41±7.01	83.69± 6.28	79.35±1.93*	20.65

*significant different from initial gallic acid content (p<0.05, ANOVA)

Table 24 Percentage contents of gallic acid remaining in serum during 12 weeks under storage condition at room temperature (mean±SD, n=3)

Formulation	Gallic acid content (%)					% Loss
	Initial	Week2	Week4	Week8	Week12	
NS2064	100	88.64±5.25	87.78±4.36	81.12 ±4.70	64.09±7.44	35.91
NS4064	100	86.93±2.25	78.83±8.88	77.53±6.50	71.15±1.29*	28.85
NS6064	100	90.44±7.77	85.91±6.96	80.95±6.63	67.09±6.35	32.91

*significant different from initial gallic acid content (p<0.05, ANOVA)

G. In vitro Permeation Study of Niosomes Loaded with Emblica Extract and Serum

In vitro percutaneous absorption methods have been used for measuring the absorption of compounds that come in contact with skin. In this study, the in vitro skin permeation study of different formulations was performed using modified Franz diffusion cell through newborn pig skin. Newborn pig skin was used because it gave similar permeation characteristics to human skin (Bronaug, Stewart and Congdon, 1982).

The determination of gallic acid content was performed by high performance liquid chromatography, HPLC because it specificity and high sensitivity. The validation of analytical method is the process which performance characteristics of the method are established to meet the requirements for the intended analytical applications. The performance characteristics are for the assay validation were specificity, linearity, accuracy and precision. The data was demonstrated in the Appendix B2.

The cumulative amounts (M) of gallic acid permeated through newborn pig skin were plotted as function of time. The permeation rate constant (Flux, J, $\mu g/cm^2 h$) of gallic acid at steady state was calculated from the slope of linear portion of the plot between cumulative amount permeated per unit area versus time. The permeation profiles of gallic acid incorporated in niosome formulations and niosome serums are depicted in Figure 30 and 31.

For further comparing and understanding the membrane permeability of these formulations, the membrane permeability coefficients (P_{app}, cm/h) were calculated. These coefficients were calculated from the slope, which obtained from the linear plot as in Figure 35. The slopes were divided by the concentration in the donor phase (C_d, $\mu g/ml$) as shown below (Reichling et al., 2006). Notably, P was determined from the diffusion coefficient (D), the partition coefficient (K) and thickness of membrane (h) which influenced to the permeability (Sinko, 2006).

$$M \quad = \quad PSC_dt$$

$$P \quad = \quad \frac{DK}{h}$$

where :

M	=	cumulative amount of gallic acid permeated (μg)
P	=	permeability coefficient (cm/h)
S	=	surface area (cm^2)
C_d	=	concentration of drug in donor (mg/ml)
t	=	time (h)
D	=	diffusion coefficient
K	=	partition coefficient
h	=	thickness of membrane

The values of flux and apparent permeation coefficient of gallic acid from various systems were calculated according Fick's law of diffusion and summarized in Table 25.
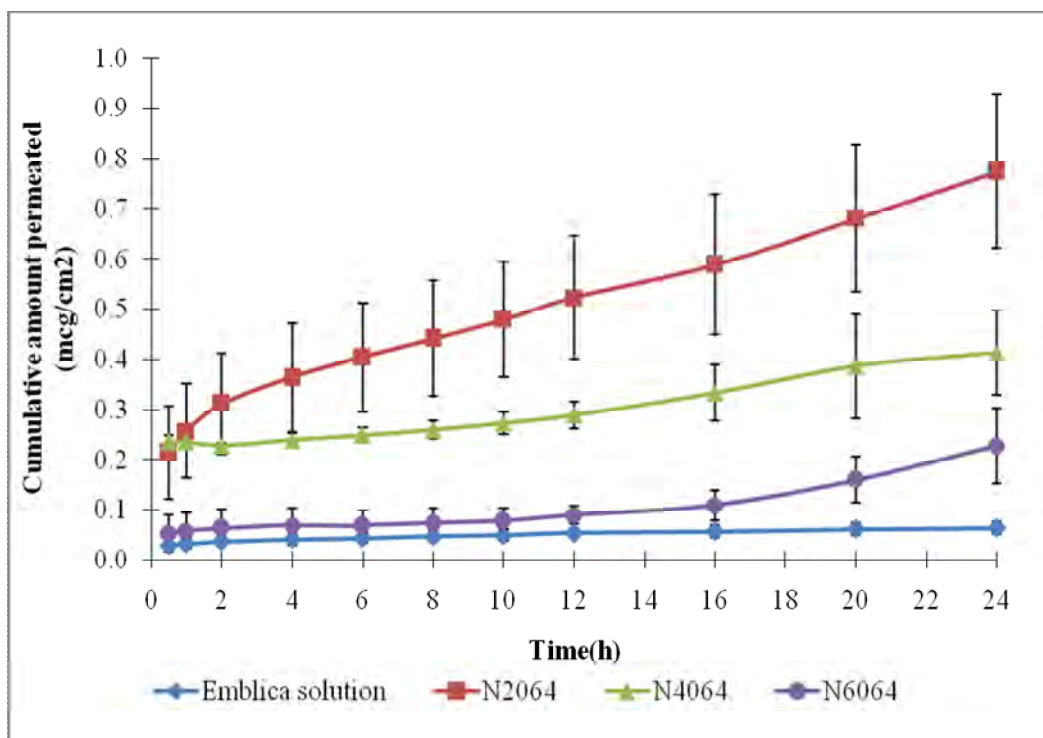
Figure 30 The skin permeation profiles of gallic acid from niosome formulations N2064, N4064, N6064 and emblica extract solution (mean±SD, n=3)
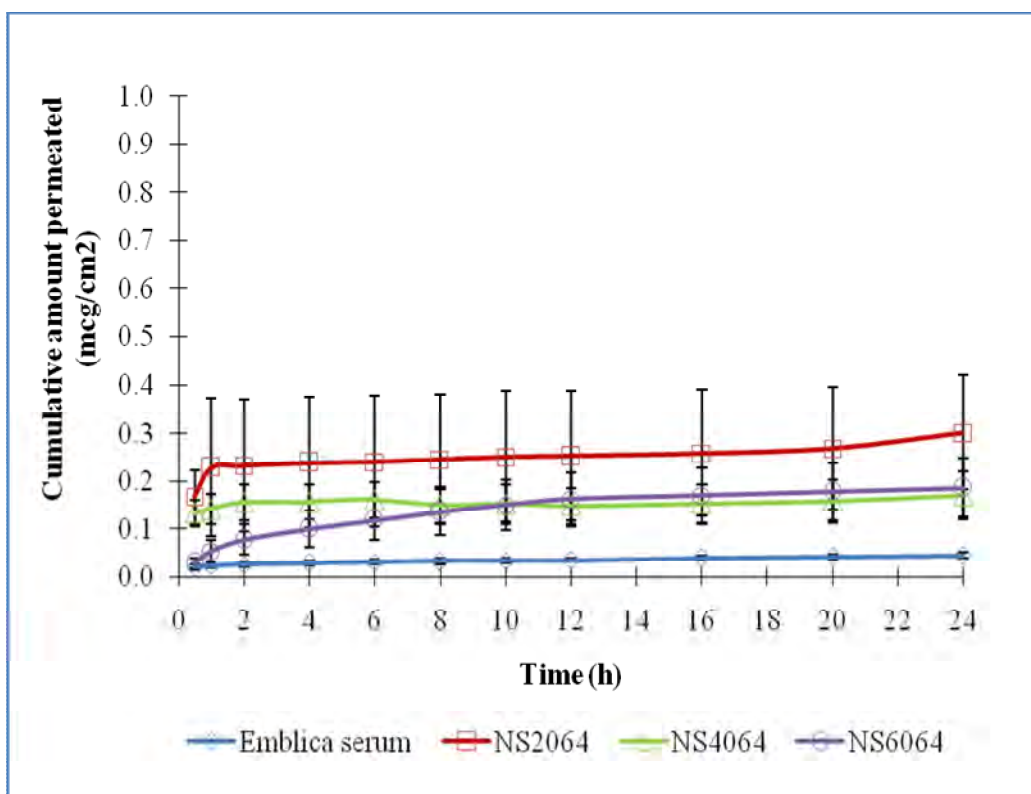
Figure 31 The skin permeation profiles of gallic acid from niosome formulations NS2064, NS4064, NS6064 and emblica extract serum (mean±SD, n=3)
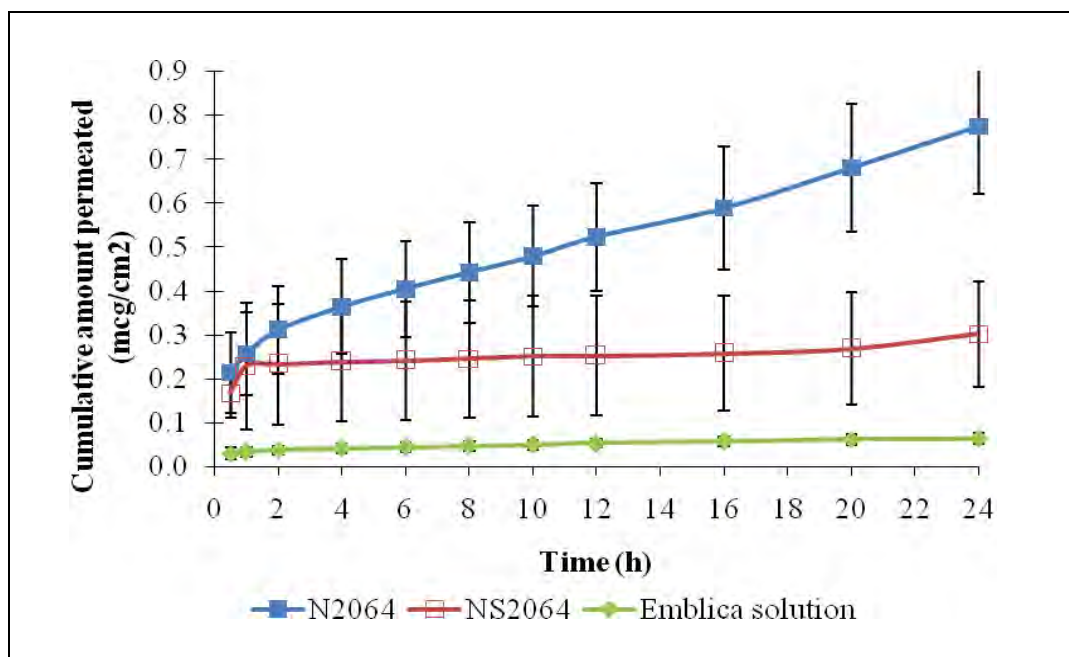
Figure 32 The skin permeation profiles of gallic acid from N2064 niosome formulation, NS2064 serum and emblica extract solution (mean±SD, n=3)
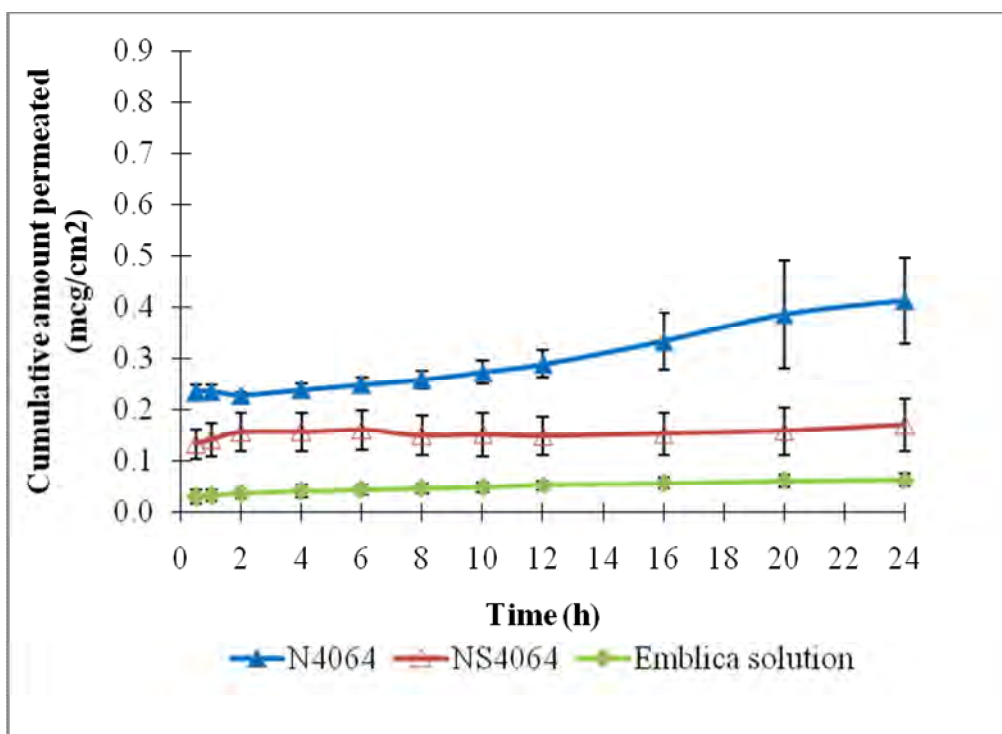
Figure 33 The skin permeation profiles of gallic acid from N4064 niosome formulation, NS4064 serum and emblica extract solution (mean±SD, n=3)
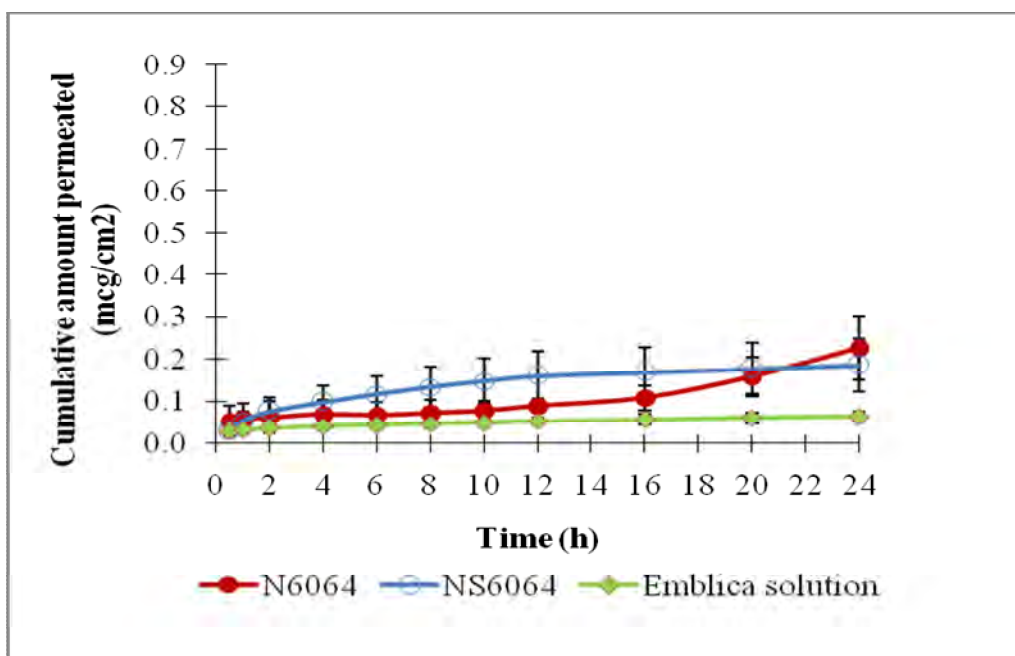
Figure 34 The skin permeation profiles of gallic acid from N6064 niosome formulation, NS6064 serum and emblica extract solution (mean±SD, n=3)

From the permeation profiles in Figures 32-34, it revealed that emblica extract loaded niosomes permeated through the model skin better than serum, emblica extract solution and emblica extract serum.

The results exhibited that niosomes vesicle behaved as carrier of gallic acid through the skin (Manosroi et al., 2010) . It could be seen that the niosome formulations N2064 had permeation higher than N4064 and N6064.

From the calculated data in Table 25 it showed that N2064 had highest flux and permeability coefficient which were 0.0070 ± 0.0026 $\mu g/cm^2/h$ and 0.0025 ± 0.0009 cm/h, respectively. Whereas, the permeation coefficient of N4064 and N6064 were not clearly different at 0.0020 and 0.0008 cm/h. This finding agreed with Ibrahim et al. (2008) that the steady state transdermal fluxes of flurbiprofen from cholesterol free proniosomal liquids of Span20 was more than those of both Span40 and Span60 cholesterol free proniosome. It might be occurred from phase transition temperature (Yoshioka et al., 1995; Mokhtar et al., 2008; Balakrishnan et al., 2009). The phase transition temperature of the vesicle systems indicated the thermodynamic state of the bilayers. If the phase transition temperature is higher than ambient temperature, the bilayer is in gel state. If the transition temperature is lower than ambient temperature, the bilayer is in liquid crystalline state.

At temperature in the experiment, molecules of Span 40 (Tc = 42ºC) and Span 60 (Tc = 53ºC) are in the ordered gel state, which the hydrocarbon chain are fully extended and closely packed. This would make the niosomal membrane more rigid and resisted to drug release. Whereas Span 20 (Tc = 16ºC) are in the liquid crystalline state, which facilitated drug release (Suwakul, Ongpipattanakul and Vardhanabhuti, 2006). Therefore the niosome prepared from Span 20 showed higher permeability than Span 40 and Span 60.

The flux and permeability coefficient of niosomes were higher than its corresponding niosome serum. However, from statistical analysis with one-way ANOVA and Dunnett T3 test at 5% significant level, there were no significant difference (p>0.05) in permeation profile among varied compositions of niosomes and niosome serums as depicted in Table G9-G10. It might be due to the variation in thickness of biological newborn pig skin used in this experiment.

In this study, the flux and the permeability coefficient of gallic acid were in range 0.0010-0.0070 $\mu g/cm^2/h$ and 0.0001-0.0025 cm/h, respectively.

In the permeation process, the niosomes were adsorbed and fused with the skin surface, which led to a high thermodynamic activity gradient of a drug at the interface (Choi and Maibach, 2005), which might partly contribute to drug permeation into the skin.
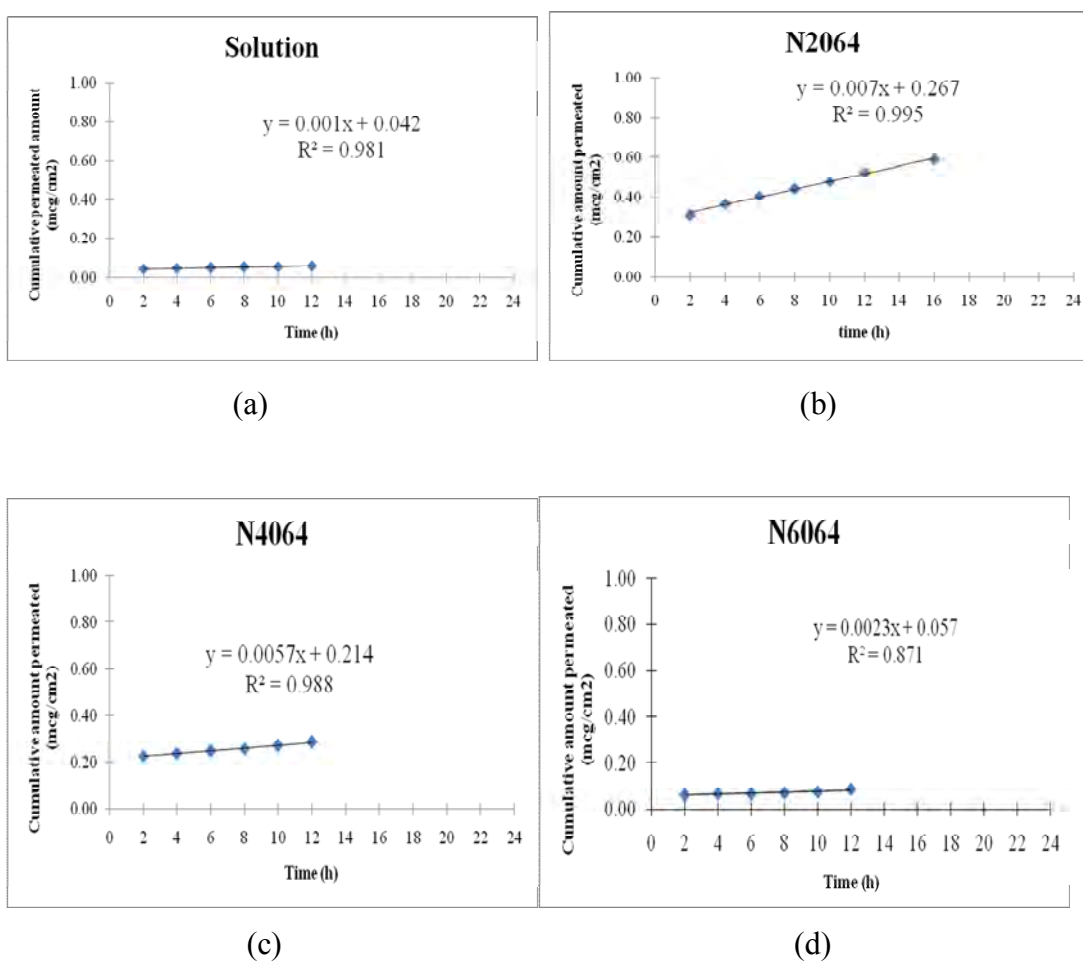
(a)   (b)

(c)   (d)

Figure 35 The permeation profile per unit area of gallic acid from (a) solution, (b) N2064, (c) N4064, (d) N6064, (e) Emblica serum, (f) NS2064, (g) NS4064 and (h) NS6064, (mean ± SD, n =3)

(e) (f)

(g) (h)
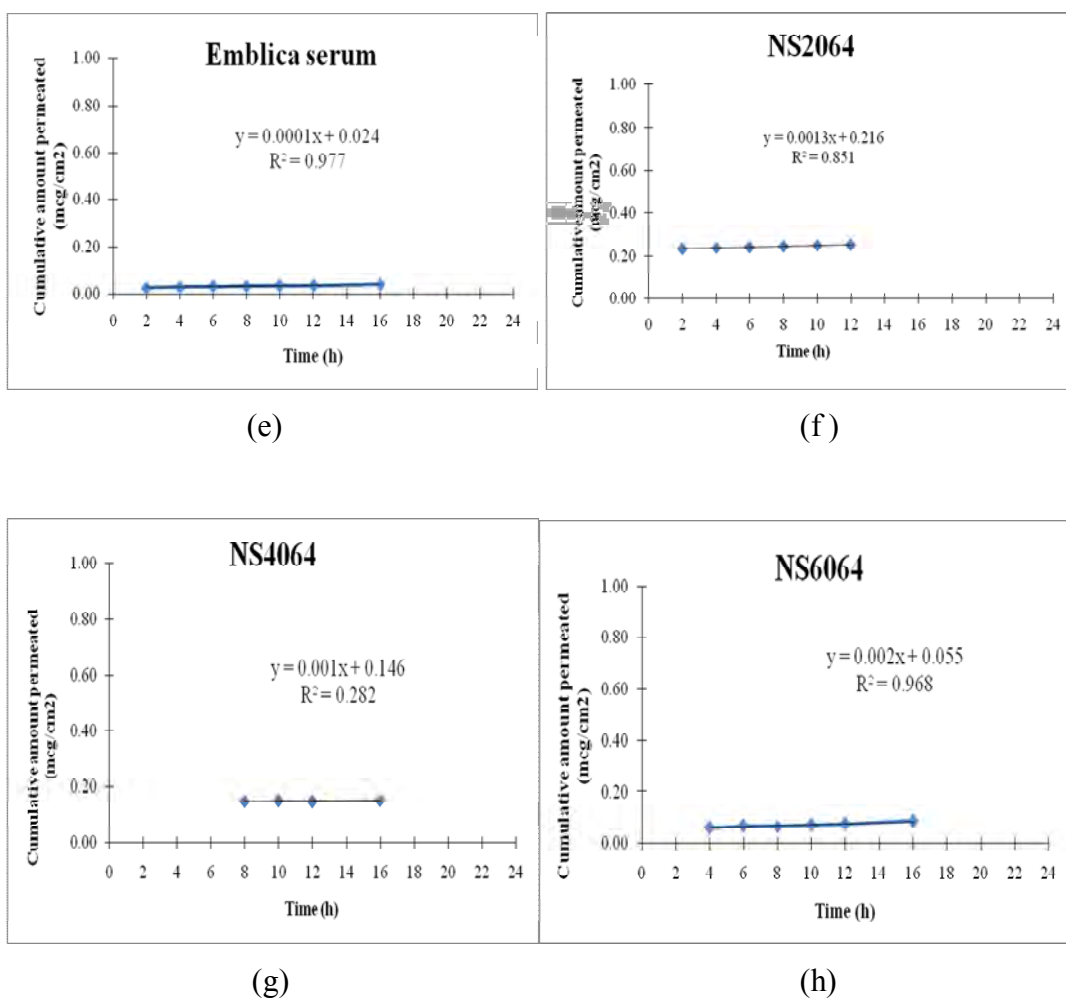
Figure 35 The permeation profile per unit area of gallic acid from (a) solution, (b) N2064, (c) N4064, (d) N6064, (e) Emblica serum, (f) NS2064, (g) NS4064 and (h) NS6064, (mean ± SD, n =3) (continued)

Table 25 The permeation rate constant, membrane permeability coefficients and percentage of drug deposited into the skin of various formulation (mean±SD, n=3)

Formulation	Slope (Flux, µg/cm^2/h)	P_{app} (cm/h)	% gallic acid deposited into the skin
Emblica solution	0.0010±0	0.0003±0	1.96±0.62
N2064	0.0070±0.0026	0.0025±0.0009	2.97±0.59
N4064	0.0057±0.0025	0.0020±0.0009	1.65±0.83
N6064	0.0023±0.0023	0.0008±0.0008	2.55±0.83
Emblica serum	0.0001±0	0.0001±0	2.04±0.76
NS2064	0.0013±0.0006	0.0010±0.0004	0.09±0.02
NS4064	0.0010±0	0.0008±0	0.39±0.34
NS6064	0.0027±0.0006	0.0020±0.0004	0.13±0.03

.

From Table 25, it was noticeable that there was an amount of gallic acid could be detected in the skin. After the experiment, the skin was assayed for the gallic acid deposited into the skin. The formulation N2064 was found to gain the highest percentage of gallic acid deposited in the skin as 2.97±0.59%, followed by the formulation N6064. This result was agreeable to the result of cumulative amount permeated through the skin. From the deposit amount of gallic acid in the skin of 2.97%, it could be calculated to be 410 µg/ml of the skin sample with dimension of 2.404 cm x 0.05 mm. This concentration was higher than that at maximal anti-free radical activity of emblica extract as shown in Figure 15.

Figure 29, the cumulative amount permeated of gallic acid from the emblica extract serum was lower than the emblica extract solution. Inaddition, the cumulative amount permeated from serums were lower than from its corresponding niosome formulations. The emblica extract serum and niosome serums composed of poloxamer 407 that formed a hydrogel by the aqueous pore of triblock copolymer that the drug released by diffusion through the extramicellar water channels of the gel matrix (Cho et al., 2010, Kapadia et al., 2009). Thus, the release rate of gallic acid from the

emblica serum and niosome serum formulations was lower than the corresponding niosome formulations and solution. This result agreed with Glavas-Dodov et al.(2003) that the percentage cumulative release of 5-fluorouracil loaded in liposome gel formulation was lower than the liposomes formulation.

As compared skin permeation of niosome formulation with the emblica extract serum, it revealed that niosomes could enhance the penetration across the skin barrier, stratum corneum. The similar result that niosomes enhanced skin permeation, could be seen from the comparison the permeation of niosomes with emblica extract solution.

From the data obtained in this study, it revealed that the application of emblica extract loaded niosomes for antioxidant and antityrosinase activity by skin delivery was feasible. In the in vitro study, the realistic factor such as mechanically application, dosage per unit area of skin was not involved. Whereas, it exhibited the important factors for skin delivery. Thus, the further in vivo investigation of niosome formulations with human subjects for antioxidative and whitening effect are needed.

CHAPTER V

CONCLUSIONS

The present study was aimed to develop niosomes containing *Phyllanthus emblica* extract. The investigation of antioxidative and antityrosinase activities of *Phyllanthus emblica* were performed by DPPH and antityrosinase assay. The new delivery system as niosomes was applied for loading emblica extract and further modified into serum. The results of this study could be concluded as follows:

1. The light brown powder of *Phyllanthus emblica* extract was obtained by soxhlet extraction and lyophilization technique to give yield as 2.53% . The gallic acid in emblica extract exhibited the same characteristics as standard gallic acid as shown by TLC. The quantitative analysis of gallic acid content in emblica extract by HPLC method was 56.29%.

2. The investigation of the hydrogen-donating activity of emblica extract, standard gallic acid and ascorbic acid was determined by DPPH scavenging activity. The emblica extract with IC_{50} of 1.38±0.01 µg/ml, showed significantly higher activity than ascorbic acid, but lower than standard gallic acid.

3. The whitening activity of emblica extract, standard gallic acid, kojic acid and alpha arbutin was determined by antityrosinase activity. The antityrosinase activity of emblica extract with IC_{50} of 1.00±0.06 mg/ml was not significantly different from gallic acid but lower than kojic acid. Kojic acid exhibited the highest antityrosinase potency, whereas the alpha arbutin showed the lowest scavenging activity.

4. Niosomes could be prepared from Span 40 and Span 60 as surfactant with all surfactant:cholesterol ratios study as 1:1, 6:4, 7:3 and Solulan C-24 as stabilizer by reverse phase evaporation method. However, Span 20 could be prepared with only two surfactant:cholesterol ratios as 1:1 and 6:4, whereas Span80 could be prepared

with only one ratio as 6:4. From the results obtained, the type of surfactant influenced the entrapment efficiency, particle size and size distribution.

5. The entrapment efficiency of gallic acid in niosome formulations prepared was ranged from 34.35-49.29%. The composition of Span20:cholesterol:Solulan C-24 as 57:38:5, gave the niosomes with the highest entrapment efficiency, 49.29%. This formulation showed the nonsignificant decrease of entrapment efficiency at the refrigerated temperature ($p<0.05$), whereas showed a significant change at room temperature.

6. At the refrigerated temperature, the loss of gallic acid in niosome formulations were between 5.83-9.26%, which was less than 10% that it was acceptable for niosomal formulation. After statistic analysis with ANOVA, there was no significant difference between niosome formulations. At the room temperature, most formulations had the loss of gallic acid content between 8.95-20.05% more than 10% loss of gallic acid content. There was no significant difference between niosome formulations ($p>0.05$). This result suggested that the appropriate storage condition for niosomes was refrigerated temperatures and protected from light.

7. Niosome serum formulations were prepared by using 16% w/v of poloxamer 407. The pH values of serums were between 3.51-3.83 which were resulted from the acidity of gallic acid. No significant change of the serums at 4 °C and room temperature for 3 months ($p>0.05$). The viscosity values were between 779-1372 centipoises, similarly no significant change of viscosity at both temperatures was found.

8. At refrigerated temperature, the degradation of gallic acid in niosome serum formulations were between 17.55-22.72%, which were in a higher degree than those occurred at 4°C. The NS4064 and NS6064 had significant decrease of gallic acid content from initial time ($p<0.05$). At room temperature, the degradation of gallic acid was between 28.85-35.91%. The NS4064 showed a significant loss of gallic acid content from initial time (ANOVA, $p<0.5$). This result might be involved with surface active property of poloxamer 407 that using as viscosity enhancer.

9. Niosome formulations loaded with emblica extract showed remarkably higher permeation through the skin than emblica extract solution. This result revealed that niosomes acted as a carrier for delivery of emblica extract into the skin. The

permeation profile of niosome formulations were higher than corresponding niosome serums. This might be attributed to the diffusion of gallic acid through the extramicellar water channels of the gel matrix.

10. The permeation of gallic acid from niosome formulation with composition of Span 20:cholesterol:Solulan C-24 of 57:38:5, exhibited the highest skin permeability coefficient of 0.0025 ± 0.0009 cm/h. The cumulative amount of gallic acid was detected in pig skin (expressed as % dose) after 24 h was $2.97\pm0.59\%$. It could be implied that niosomes with emblica extract might exhibit its antioxidative effects in the skin.

However, further studies regarding investigation the mechanism of transdermal delivery, in vivo test of efficacy in human subject may be performed to confirm the result of this study in the future.

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APPENDICES

APPENDIX A

Molecular structure and physical properties of material

1.   **Cholesterol**

    Molecular formula $C_{27}H_{46}O$

    Molecular weight   386.65

    Structure
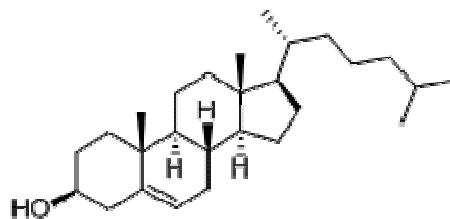
2.   **Span® 20**

    Chemical name   Sorbitan laurate; Sorbitan monododecanoate

    Molecular formula $C_{18}H_{34}O_6$

    Molecular weight   346

    Transition temperature   16 ºC

    HLB   8.6

    Structure
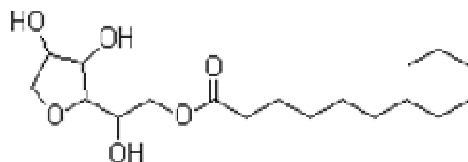
3.   **Span® 40**

    Chemical name   Sorbitan monopalmitate

    Molecular formula $C_{22}H_{42}O_6$

    Molecular weight   403

    Transition temperature   42 ºC

    HLB   6.7
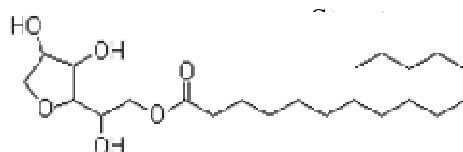
4.   Span® 60

Chemical name   Sorbitan monooctadecanoate; Sorbitan stearate

Molecular formula $C_{24}H_{46}O_6$

Molecular weight   431

Transition temperature   53 ºC

HLB   4.7
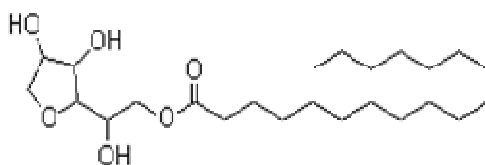
Structure

5.   Span® 80

Chemical name   Sorbitan oleate

Molecular formula $C_{24}H_{44}O_6$

Molecular weight   429

Transition temperature   -20.3 ºC

HLB   4.3
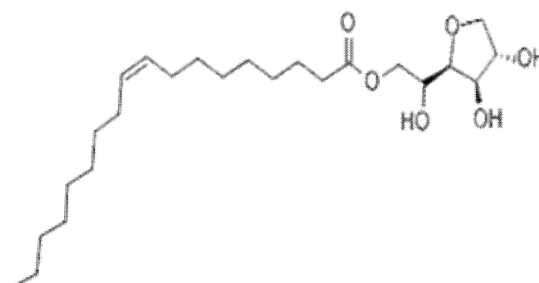
Structure

6. **Solulan® C-24**

    Chemical name   polyoxyethylene-24-cholesteryl ether
    Molecular weight   1443
    Structure
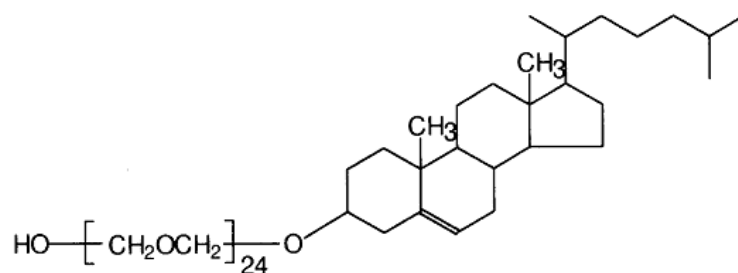
7. **Poloxamer 407**

    Chemical name   a-Hydro-o-hydroxypoly(oxyethylene)poly
                           (oxypropylene)poly(oxyethylene)block copolymer
    Molecular formula $C_5H_{10}O_2$
    Molecular weight   102.132
    Structure
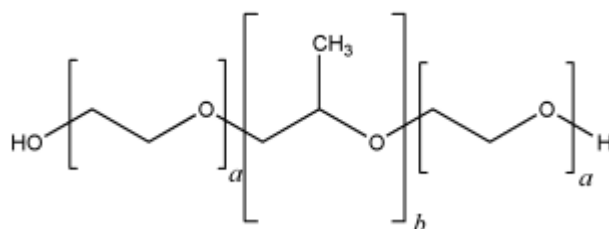
APPENDIX B

Validation of analytical method

Analysis of gallic acid by high performance liquid chromatographic (HPLC) method

    1.   The determination of gallic acid content in isopropanol and diluted with methanol and mobile phase

        The determination of amount of entrapped gallic acid was performed by HPLC method due to it sensitivity and accuracy. The validation of analytical method is the process by which it is established that the performance characteristics of the method meet the requirements for the intended analytical applications. The performance characteristics are expressed in terms of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

    1.1. Specificity

        The specificity of an analytical method is the ability to measure the analyses accurately and with specificity in the presence of other components in the sample. Figure B1 showed typical chromatograms of standard gallic acid and paracetamol, niosomes systems and niosome serum systems, respectively. The chromatograms of standard gallic acid and the internal standard were exactly the same as in the system used methanol and diluted with mobile phase as solvent. All surfactants and gelling agent with used in the niosomes showed no peaks in the condition used. Thus, the HPLC conditions used in the study had suitable specificity.
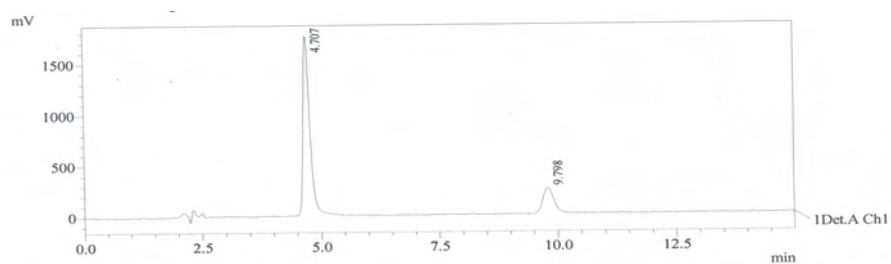
(a) standard gallic acid and paracetamol in isopropanol and diluted with methanol and mobile phase
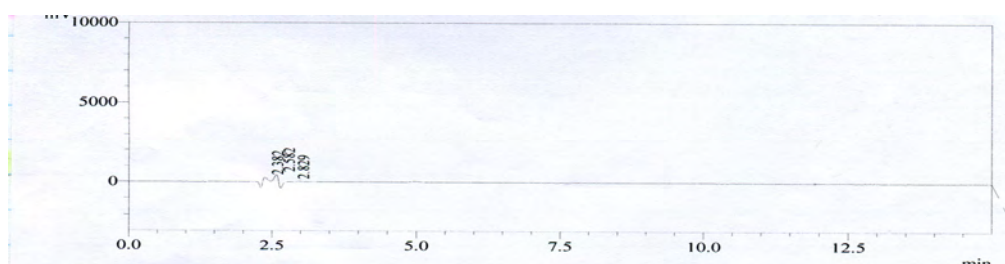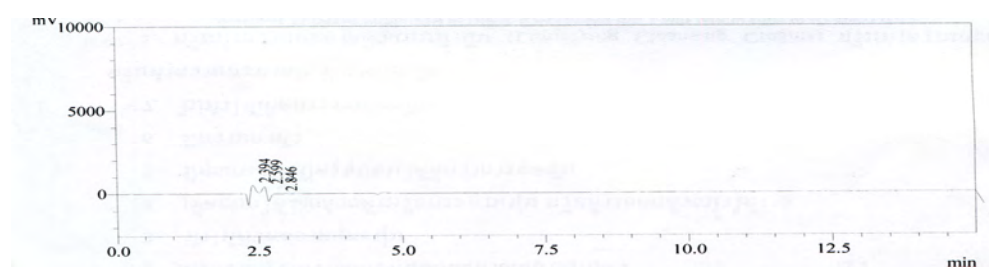
(b) Span 20 niosomes
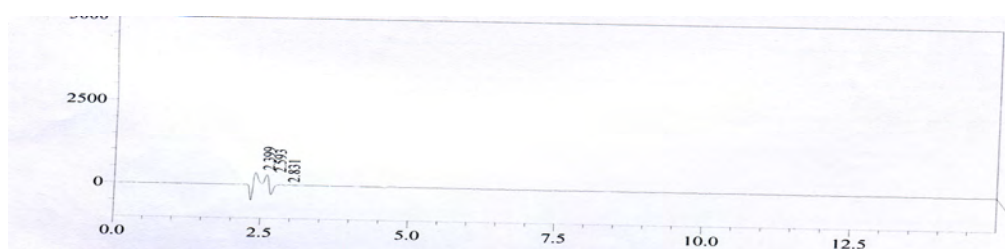
(c) Span 40 niosomes

(d) Span 60 niosomes

Figure B1 HPLC chromatograms of in HPLC validation (a) standard gallic acid and paracetamol in isopropanol and diluted with methanol and mobile phase, (b) Span 20 niosomes, (c) Span 40 niosomes, (d) Span 60 niosomes, (e) Span 80 niosomes, (f) Span 20 niosome serum, (g) Span 40 niosome serum and (h) Span 60 niosome serum (continued)
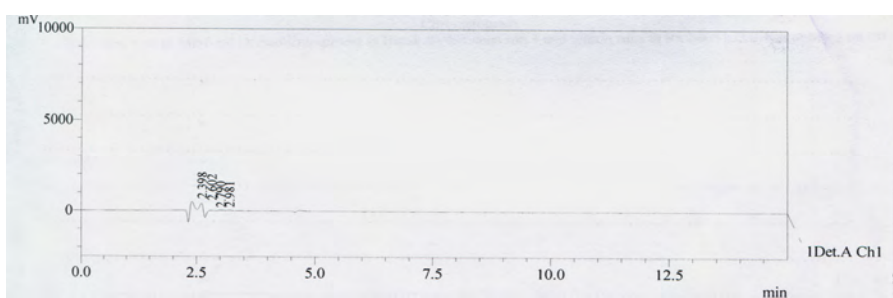
(e) Span80 niosomes
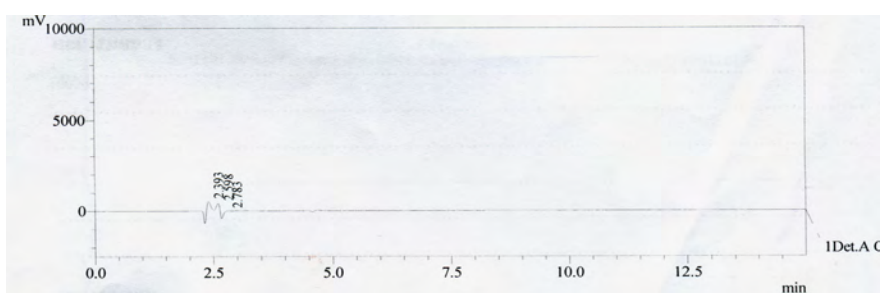
(f) Span20 niosome serum
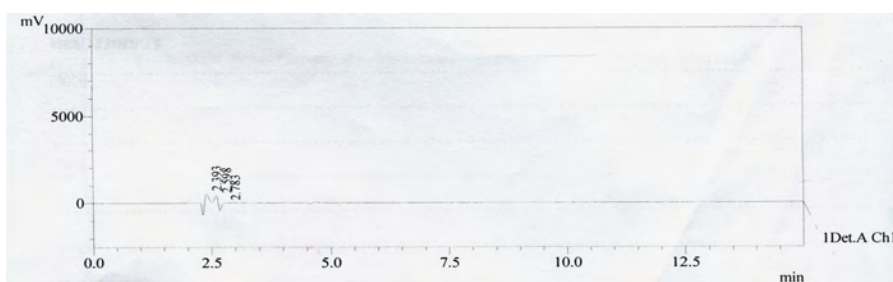
(g) Span40 niosome serum
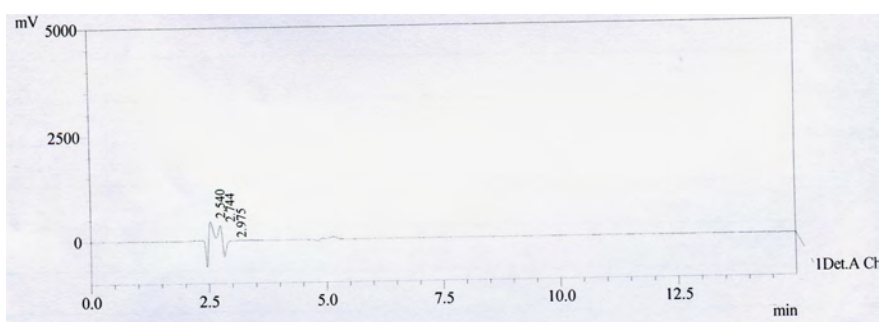
(h) Span60 niosome serum

Figure B1 HPLC chromatograms of in HPLC validation (a) standard gallic acid and paracetamol in isopropanol and diluted with methanol and mobile phase, (b) Span 20 niosomes, (c) Span 40 niosomes, (d) Span 60 niosomes, (e) Span 80 niosomes, (f) Span 20 niosome serum, (g) Span 40 niosome serum and (h) Span 60 niosome serum (continued)

1.2. **Linearity**

Data of the standard gallic acid concentration and peak area ratio of the standard gallic acid concentrations in isoporopanol and diluted with methanol and mobile phase and in the presence of all surfactants used in the niosomes systems (Span60) and niosome serum systems (Span60) are depicted in Tables B1. The good linearity was shown with R^2 of 0.9999

Table B1 Data for calibration curve of gallic acid in isopropanol and diluted with methanol and mobile phase

Concentration (µg/ml)	Peak area ratio			Mean	SD	%CV
	Set 1	Set 2	Set 3			
0.16	0.6300	0.6219	0.6463	0.6327	0.0124	1.96
0.32	1.6056	1.5937	1.6433	1.6142	0.0259	1.60
0.80	4.5345	4.7130	4.6396	4.6290	0.0897	1.94
1.60	9.6026	9.8491	9.5342	9.6620	0.1656	1.71
2.40	14.6798	14.6556	14.5145	14.6166	0.0893	0.61
3.20	19.3789	19.9854	19.7868	19.7170	0.3092	1.57
R^2	0.9999	0.9998	0.9998	0.9999	-	-
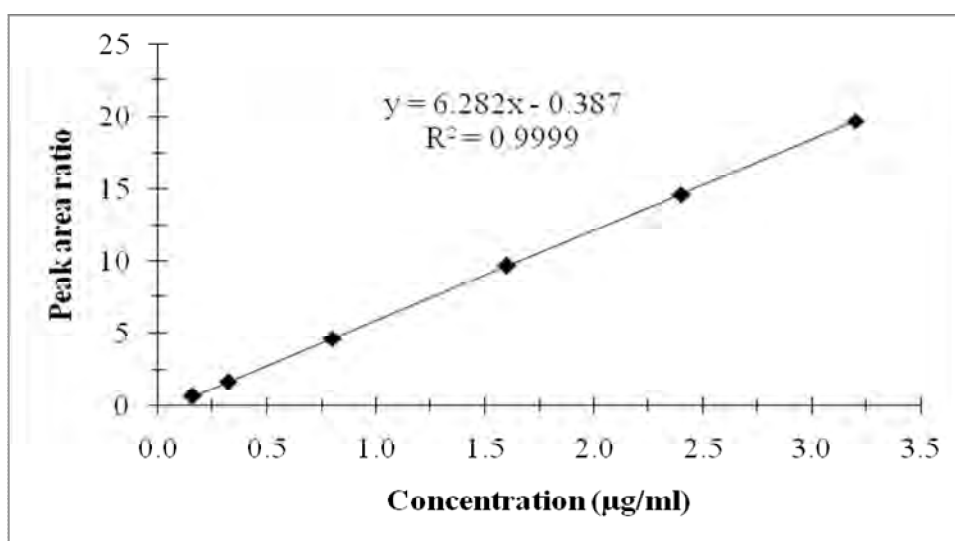
Figure B2 Calibration curve of gallic acid in methanol and mobile phase by HPLC method

123

1.3. **Accuracy**

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. Accuracy is calculated as percent recovery by the assay of known added amount of analyses. The percentages of analytical recovery of gallic acid solution are shown in Tables B3. The percentages analytical recovery of gallic acid were in range 100±2% of which indicated that this method could be used for analysis in all concentrations studies with high accuracy.

Table B2 The percentages of analytical recovery of low, medium and high concentrations of gallic acid in isopropanol and diluted with methanol and mobile phase by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
0.24	0.2435	101.27	
0.24	0.2389	99.36	
0.24	0.2410	100.24	99.36 ± 1.02
0.24	0.2377	98.90	
0.24	0.2374	98.90	
1.20	1.1995	99.79	
1.20	1.1950	99.42	
1.20	1.2237	101.80	99.70 ± 1.27
1.20	1.1842	98.52	
1.20	1.1895	98.96	
2.80	2.7925	99.79	
2.80	2.7708	98.79	
2.80	2.8028	99.93	100.17 ± 1.06
2.80	2.8258	100.75	
2.80	2.8494	101.60	

Table B3 The percentages of analytical recovery of low, medium and high concentrations of gallic acid in N6011 Niosomes by by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
0.24	0.2432	101.33	
0.24	0.2410	100.41	
0.24	0.2427	101.14	100.08 ± 0.52
0.24	0.2402	100.10	
0.24	0.2424	101.01	
1.20	1.1864	98.87	
1.20	1.1768	98.07	
1.20	1.1845	98.71	98.84 ± 0.59
1.20	1.1965	99.71	
1.20	1.1881	99.01	
2.80	2.7507	98.24	
2.80	2.7541	98.36	
2.80	2.7530	98.32	99.23 ± 1.43
2.80	2.8437	101.56	
2.80	2.7913	99.69	

Table B4 The percentages of analytical recovery of low, medium and high concentrations of gallic acid in NS6011 Niosome serum by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
0.24	0.2438	101.58	
0.24	0.2441	101.70	
0.24	0.2427	101.12	100.27 ± 1.66
0.24	0.2369	98.70	
0.24	0.2358	98.24	
1.20	1.1779	98.16	
1.20	1.1801	98.34	
1.20	1.1819	98.49	98.88 ± 1.14
1.20	1.2108	100.90	
1.20	1.1821	98.51	
2.80	2.7717	98.99	
2.80	2.7540	98.34	
2.80	2.7529	98.01	98.90 ± 0.91
2.80	2.8436	100.39	
2.80	2.7913	98.78	

1.4. Precision

The precision of gallic acid analyzed by HPLC method were detrmined both within run precision and between run precisions as illustration in Table B6 and B7. The coefficients of variation values were 1.06-1.47% and 0.39-0.93%, respectively. The coefficient of variation of an analytical method should generally be less than 2%. Therefore, the HPLC method was precise for quantitative analysis of gallic acid in the range studies.

Table B5 Data of within run precision of gallic acid isopropanol and diluted with methanol and mobile phase by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)					Mean	SD	%CV
	1	2	3	4	5			
0.24	0.2435	0.2389	0.2410	0.2377	0.2374	0.2397	0.0026	1.06
1.20	1.1995	1.195	1.2237	1.1842	1.1895	1.1984	0.0176	1.47
2.80	2.7925	2.7708	2.8028	2.8258	2.8494	2.8083	0.0304	1.08

Table B6 Data of between run precision of gallic acid isopropanol and diluted with methanol and mobile phase by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)				Mean	SD	%CV
	1	2	3	4			
0.24	0.2435	0.2413	0.2420	0.2429	0.2424	0.0010	0.39
1.20	1.1995	1.1751	1.1971	1.1872	1.1897	0.0111	0.93
2.80	2.7925	2.8323	2.8056	2.7900	2.8051	0.0194	0.69

2. The determination of gallic acid content in phosphate buffer pH7.4

The determination of gallic acid content in phosphate buffer pH7.4 was performed for analysis of gallic content in receiver medium of Franz cell in permeation study.

The validation of analytical method is the process by which it is established that the performance characteristics of the method meet the requirement for the intended analytical applications. The performance characteristics are expressed in term of analytical parameters. For HPLC assay validation, these include specificity, linearity, accuracy and precision.

2.1 Specificity

The specificity of an analytical method is the ability to meet analyses accurately and with specificity in the presence of other components in the sample. The chromatogram of standard gallic acid shown in Figure B3. The chromatogram demonstrated that the HPLC condition used in the study had a suitable specificity.
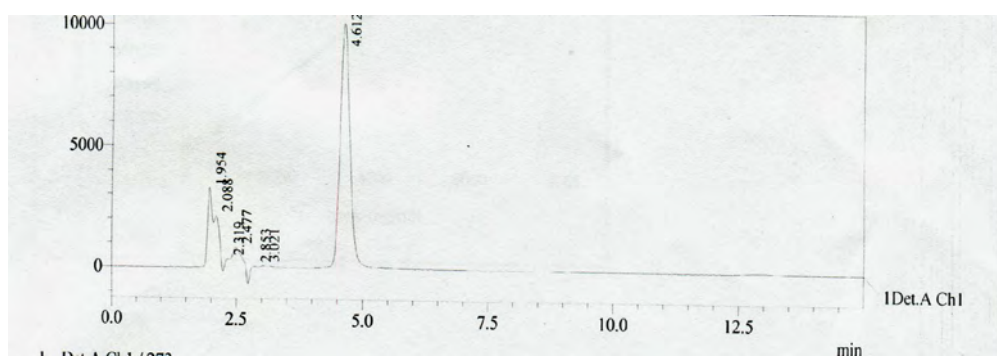
(a)   standard gallic acid in phosphate buffer pH 7.4
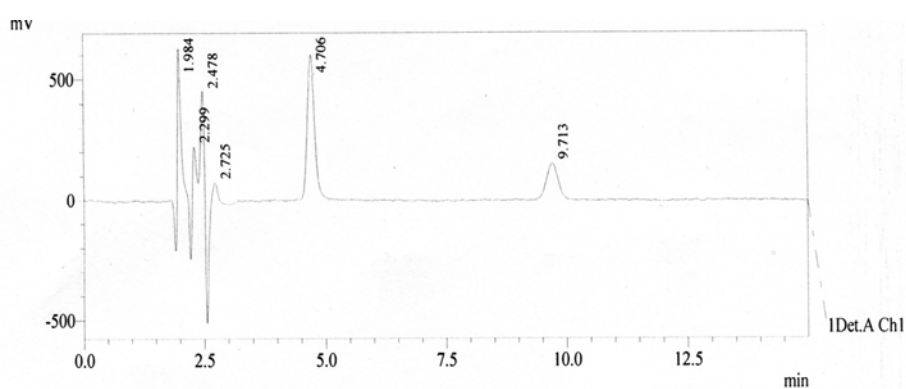
(b)   standard gallic acid and paracetamol in phosphate buffer pH 7.4

Figure B3   HPLC chromatograms of in HPLC validation, (a) standard gallic acid in phosphate buffer pH 7.4, (b) standard gallic acid and paracetamol in phosphate buffer pH 7.4

2.2 Linearity

Table B7 Data for calibration curve of gallic acid in PBS pH7.4

Concentration (μg/ml)	Peak area ratio			Mean	SD	%CV
	Set 1	Set 2	Set 3			
0.082	0.7575	0.7746	0.7536	0.7619	0.0112	1.47
0.164	1.1970	1.1945	1.1574	1.1830	0.0222	1.87
0.412	3.3085	3.3514	3.3619	3.3406	0.0283	0.85
2.058	22.9044	23.5146	23.8011	23.4067	0.4580	1.96
8.232	97.3124	97.1452	95.6401	96.6992	0.9210	0.95
16.464	198.5000	197.1844	194.8348	196.8397	1.8567	0.94
R^2	0.9999	0.9999	0.9999	0.9999	-	-
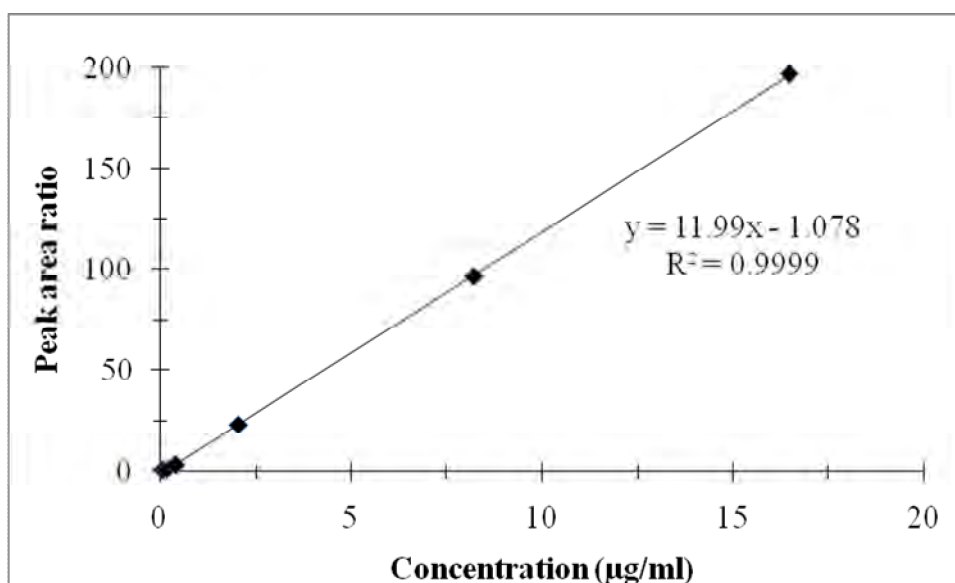
Figure B4 Calibration curve of gallic acid in phosphate buffer pH 7.4 by HPLC method

2.2 Accuracy

The accuracy of an analytical method is the closeness test results obtained by the method to the true value. Accuracy is calculated as percent recovery by the assay of known added amount of analyses. The percentages of analytical recovery of gallic acid solution are shown in Table B9.

Table B8 The percentages of analytical recovery of low, medium and high concentrations of gallic acid in phosphate buffer pH 7.4 by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
0.24	0.2504	101.42	
0.24	0.2425	98.22	100.80 ± 0.52
0.24	0.2428	98.34	
0.82	0.8208	99.71	
0.82	0.8267	100.43	99.68 ± 0.76
0.82	0.8142	98.91	
12.35	12.2487	99.20	
12.35	12.3701	100.18	99.55 ± 0.55
12.35	12.2566	99.26	

2.3 Precision

The precision of gallic acid analyzed by HPLC method was determined both within run precision and between run precision as illustrated in Table B10 and Table B11. All coefficients of variantion values were small, as 0.55-1.06% and 0.48-1.47%, respectively. The coefficient of variation of an anlytical methos should generally be less than 2%. Therefore, the HPLC method was precise for quantitative analysis of gallic acid in the range studied.

Table B9 Data of within run precision of gallic acid in PBS pH 7.4 by HPLC method

concentration (µg/ml)	estimated concentration (µg/ml)			mean	SD	%CV
	1	2	3			
0.2469	0.2504	0.2425	0.2428	0.2452	0.0026	1.06
0.8232	0.8208	0.8267	0.8142	0.8206	0.0063	0.76
12.3480	12.2487	12.3701	12.2566	12.2918	0.0679	0.55

Table B10 Data of between run precision of gallic acid in PBS pH 7.4 by HPLC method

concentration (µg/ml)	estimated concentration (µg/ml)			mean	SD	%CV
	1	2	3			
0.2469	0.2428	0.2448	0.2498	0.2458	0.0036	1.47
0.8232	0.8372	0.8208	0.8201	0.8260	0.0097	1.17
12.3480	12.2487	12.2316	12.1399	12.2067	0.0585	0.48

3. The determination of gallic acid content in methanol

The determination of gallic acid content on methanol was performed for analysis of gallic acid content in new born pig skin in permeation study.

Table B11 Data for calibration curve of gallic acid in methanol by HPLC method

Concentration (µg/ml)	Peak area ratio			Mean	SD	%CV
	Set 1	Set 2	Set 3			
0.082	0.6902	0.6761	0.6998	0.6887	0.0119	1.73
0.164	0.9874	1.0107	0.9768	0.9916	0.0173	1.75
0.412	2.9822	2.9122	2.8878	2.9274	0.0490	1.67
2.058	21.3305	20.9220	21.3014	21.1846	0.2279	1.08
8.232	87.0918	89.5355	89.5715	88.7329	1.4214	1.60
16.464	171.8591	175.3067	175.6702	174.2787	2.1033	1.21
R^2	0.9999	0.9998	0.9998	0.9998	-	-
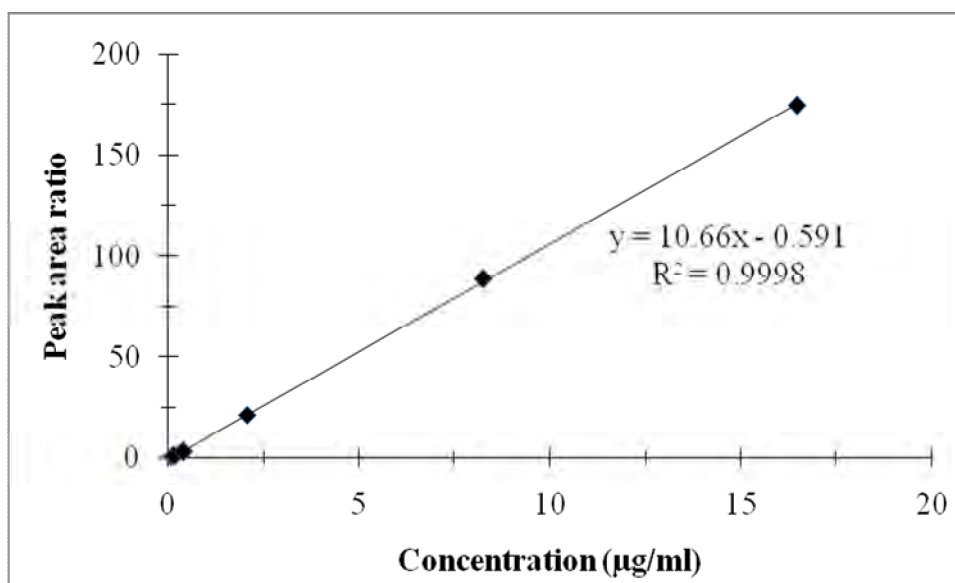
Figure B5 Calibration curve of gallic acid in methanol by HPLC method

3.2 Accuracy

The accuracy of an analytical method is the closeness test results obtained by the method to the true value. Accuracy is calculated as percent recovery by the assay of known added amount of analyses. The percentages of analytical recovery of gallic acid solution are shown in Table B13.

Table B12 The percentages of analytical recovery of low, medium and high concentrations of gallic acid in methanol by HPLC method

Concentration (µg/ml)	Estimated concentration (µg/ml)	% Accuracy	Mean ± SD
0.24	0.2504	101.42	
0.24	0.2425	98.22	99.32 ± 1.81
0.24	0.2428	98.34	
0.82	0.8208	99.71	
0.82	0.8267	100.43	99.68 ± 0.76
0.82	0.8142	98.91	
12.35	12.2487	99.20	
12.35	12.3701	100.18	99.54 ± 0.55
12.35	12.2566	99.26	

2.3 Precision

The precision of gallic acid analyzed by HPLC method was determined both within run precision and between run precision as illustrated in Table B14 and Table B15. All coefficients of variantion values were small, as 0.48-1.47% and 0.83-1.25%, respectively. The coefficient of variation of an anlytical methos should generally be less than 2%. Therefore, the HPLC method eas precise for quantitative analysis of gallic acid in the range studied.

Table B13 Data of within run precision of gallic acid in methanol by HPLC method

concentration (µg/ml)	estimated concentration (µg/ml)			mean	SD	%CV
	1	2	3			
0.2469	0.2428	0.2448	0.2498	0.2458	0.0036	1.47
0.8232	0.8372	0.8208	0.8201	0.8260	0.0097	1.17
12.3480	12.2487	12.2316	12.1399	12.2067	0.0585	0.48

Table B14 Data of between run precision of gallic acid in methanol by HPLC method

concentration (µg/ml)	estimated concentration (µg/ml)			mean	SD	%CV
	1	2	3			
0.2469	0.2504	0.2473	0.2442	0.2473	0.0031	1.25
0.8232	0.8269	0.8067	0.8156	0.8164	0.0101	1.24
12.3480	12.4458	12.3935	12.2476	12.3623	0.1027	0.83

APPENDIX C

Determination of Hydrogen-donating Activity

(DPPH Radical Scavenging Activity)

Table C1 The data for DPPH radical inhibition percentages of the emblic extract

No.	Concentration (µg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	0.26	10.15	14.02	17.45	13.87	3.65
3	0.51	22.65	19.34	26.48	22.82	3.57
4	0.77	29.29	31.36	39.25	33.30	5.26
5	1.02	37.04	42.41	49.45	42.97	6.23
6	1.53	45.41	57.69	61.25	54.78	8.31
7	2.04	66.71	77.25	77.72	73.89	6.22
8	2.56	83.99	86.36	81.91	84.09	2.23
9	3.07	92.92	93.81	94.15	93.63	0.64
10	4.09	93.98	93.67	92.90	93.52	0.56
11	5.11	94.02	94.31	94.25	94.19	0.15
12	7.67	93.88	93.87	93.38	93.71	0.29
13	10.22	93.52	94.13	94.40	94.02	0.45
14	30.66	93.94	93.99	94.15	94.03	0.11

Table C2 The data for DPPH radical inhibition percentages of gallic acid

No.	Concentration (μg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0.00	0.00	0.00	0.00	0.00	0.00
2	0.27	18.92	27.96	25.21	24.03	4.63
3	0.58	38.32	48.88	48.66	45.29	6.04
4	0.80	44.63	60.51	53.62	52.92	7.96
5	1.06	57.84	69.37	60.27	62.49	6.07
6	1.59	91.93	93.34	91.16	92.15	1.10
7	2.65	92.48	93.79	94.06	93.44	0.85
8	3.18	93.22	93.89	94.12	93.74	0.47
9	5.30	93.22	93.65	93.78	93.55	0.30
10	10.60	94.27	94.53	94.37	94.39	0.13
11	31.80	93.74	93.55	94.25	93.85	0.36

Table C3 The data for DPPH radical inhibition percentages of ascorbic acid

No.	Concentration ($\mu g/ml$)	% inhibition			mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	0.31	9.01	7.25	7.85	8.04	0.90
3	0.62	18.60	16.38	14.33	16.44	2.13
4	0.93	18.39	21.66	19.57	19.87	1.65
5	1.24	35.35	32.70	26.99	31.68	4.27
6	1.85	39.19	42.81	41.59	41.20	1.84
7	3.09	72.85	77.13	71.77	73.92	2.83
8	3.11	90.58	90.70	88.86	90.05	1.03
9	6.18	95.99	95.78	96.41	96.06	0.32
10	12.36	98.69	98.80	98.80	98.76	0.06
11	37.08	98.65	99.32	99.11	99.03	0.34

Table C4 Test of homogeneity of variance on the IC_{50} values of DPPH radical inhibition

Test of Homogeneity of Variances

IC50

Levene Statistic	df1	df2	Sig.
.636	2	6	.562

Table C5 One-way analysis of variance on the IC_{50} values of DPPH radical inhibition

ANOVA

IC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.711	2	1.355	125.741	.000
Within Groups	.065	6	.011		
Total	2.776	8			

Table C6 Multiple Comparisons on the IC_{50} values of DPPH radical inhibition by Tukey method

Multiple Comparisons

Dependent Variable : IC50

(I) categories	(J) categories	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
vitamin C	gallic acid	1.3437333*	.0847744	.000	1.083622	1.603844
	emblica extract	.7076000*	.0847744	.000	.447489	.967711
gallic acid	Ascorbic acid	-1.3437333*	.0847744	.000	-1.603844	-1.083622
	emblica extract	-.6361333*	.0847744	.001	-.896244	-.376022
emblica extract	Ascorbic acid	-.7076000*	.0847744	.000	-.967711	-.447489
	gallic acid	.6361333*	.0847744	.001	.376022	.896244

*. The mean difference is significant at the 0.05 level.

Table C7 Homogeneous subset on the IC_{50} values of DPPH inhibition by Tukey method

IC50

Tukey HSD[a]

categories	N	Subset for alpha = 0.05 1	Subset for alpha = 0.05 2	Subset for alpha = 0.05 3
gallic acid	3	.744933		
emblica extract	3		1.381067	
Ascorbic acid	3			2.088667
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Emblica extract (n₁)

$$y = 29.33x + 5.429$$
$$R^2 = 0.9899$$

Emblica extract (n₂)

$$y = 30.09x + 8.563$$
$$R^2 = 0.9779$$

Emblica extract (n₃)

$$y = 26.81x + 16.57$$
$$R^2 = 0.9682$$
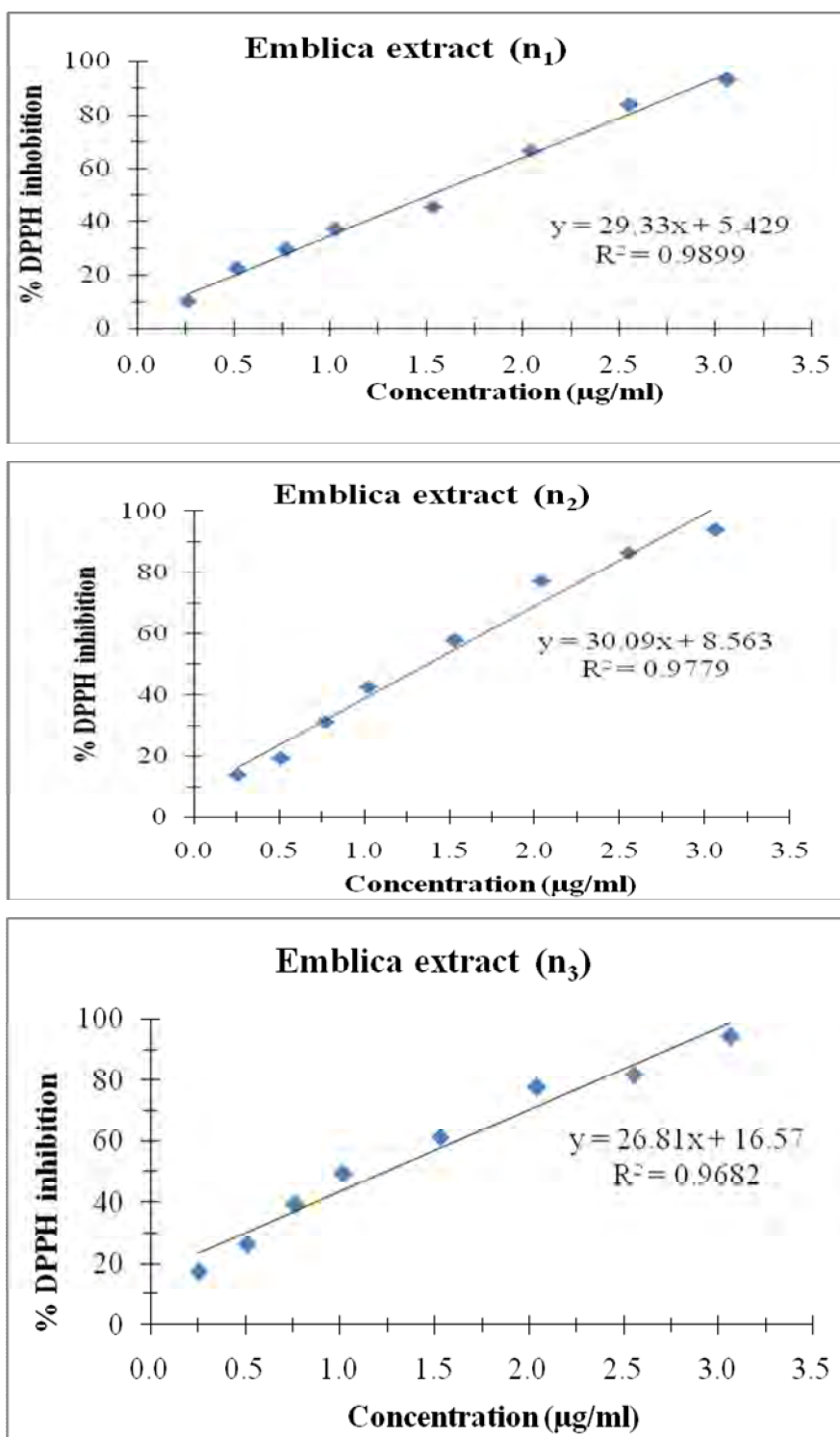
Figure C1 The relation of the % DPPH inhibition-concentration profile of emblica extract
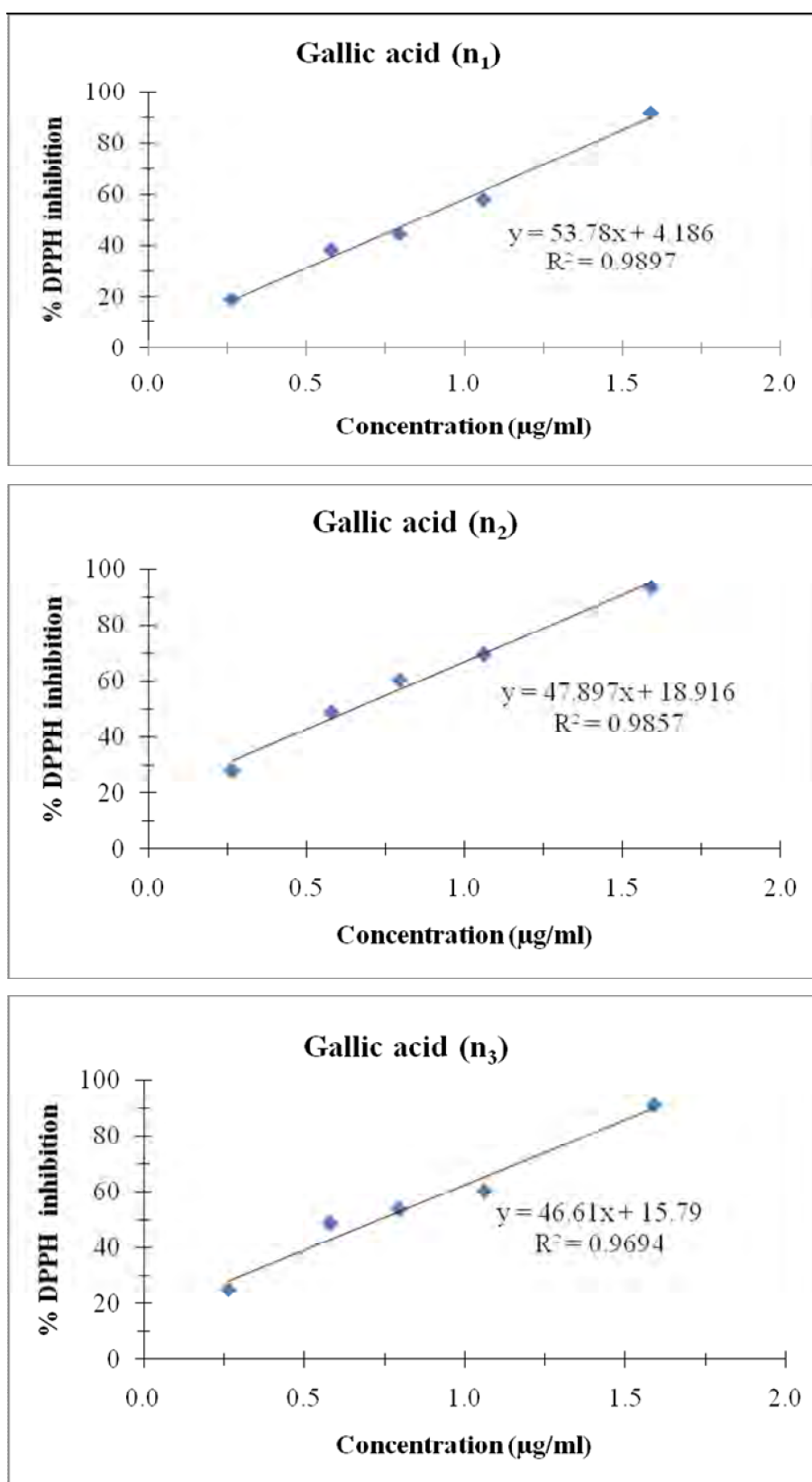
Figure C2 The relation of the % DPPH inhibition-concentration profile of gallic acid
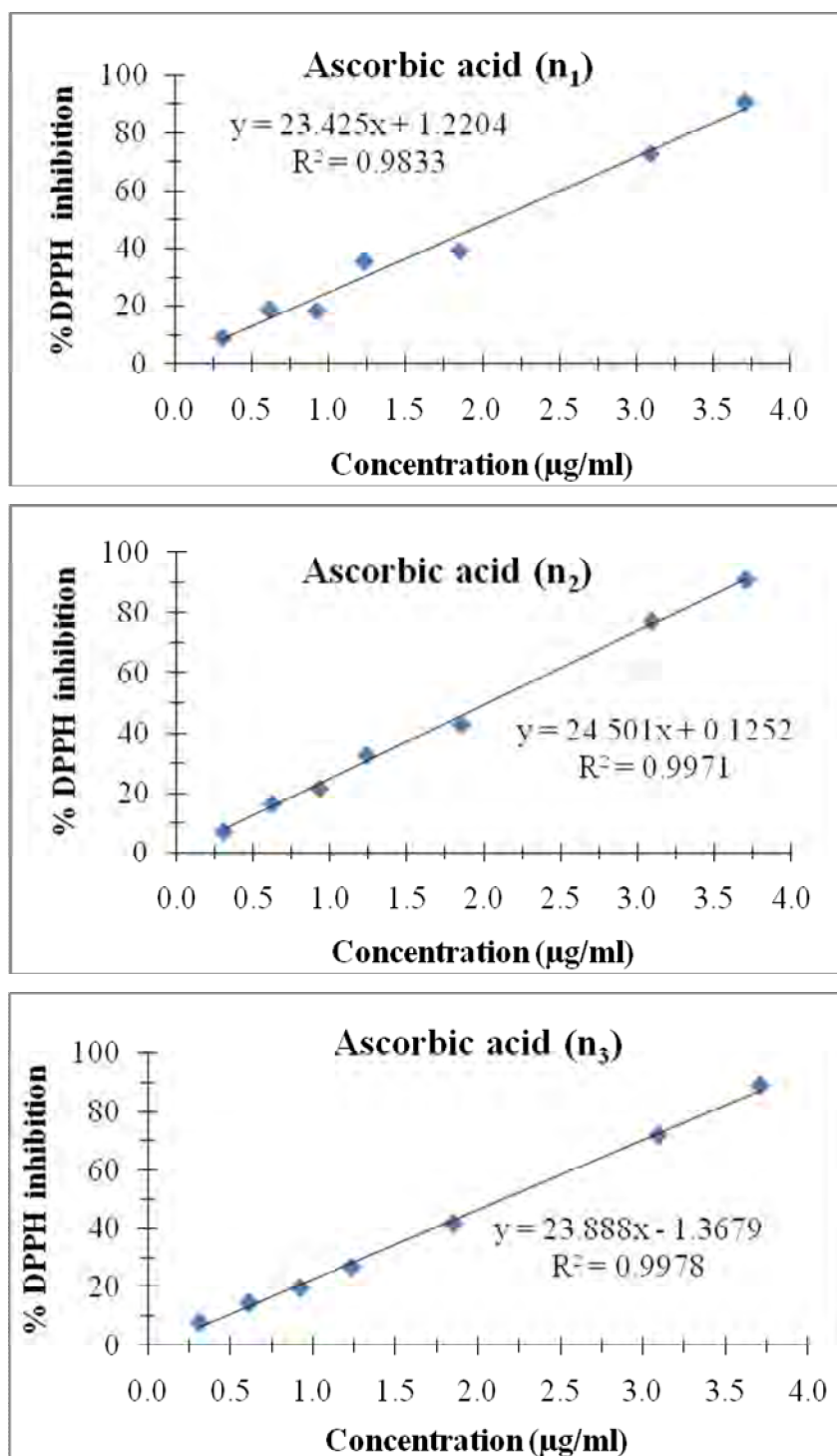
Figure C3 The relation of the % DPPH inhibition-concentration profile of ascorbic acid

APPENDIX D

Determination of Antityrosinase Activity

Table D1 The data for tyrosinase inhibition percentages of emblica extract

No.	Concentration (mg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	0.789	32.13	27.54	22.74	27.47	4.70
3	1.183	77.70	66.89	69.78	71.46	5.60
4	1.577	85.90	87.54	86.29	86.58	0.86
5	1.972	87.54	89.84	88.16	88.51	1.19
6	2.366	91.48	89.84	92.52	91.28	1.35

Table D2 The data for tyrosinase inhibition percentages of gallic acid

No.	Concentration (mg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	0.394	13.44	15.61	6.54	11.86	4.74
3	0.790	49.84	42.86	33.66	42.12	8.11
4	1.184	69.51	73.42	69.61	70.85	2.23
5	1.579	82.62	76.74	81.05	80.14	3.04
6	1.974	75.74	80.73	81.70	79.39	3.20
7	2.369	82.95	83.06	80.72	82.24	1.32

Table D3 The data for tyrosinase inhibition percentages of kojic acid

No.	Concentration (µg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	0.0083	8.52	8.26	24.68	13.82	9.40
3	0.0236	20.45	23.60	40.78	28.28	10.94
4	0.0310	39.49	30.97	42.08	37.51	5.81
5	0.0566	62.50	56.64	61.56	60.23	3.15
6	0.0746	77.56	74.63	78.18	76.79	1.90
7	0.0864	93.18	86.43	88.31	89.31	3.48
8	0.0864	95.17	86.43	95.84	92.48	5.25

Table D4 The data for tyrosinase inhibition percentages of alpha arbutin

No.	Concentration (mg/ml)	% inhibition			Mean	SD
		N1	N2	N3		
1	0	0	0	0	0	0
2	24.16	4.72	5.25	11.29	7.09	3.65
3	36.24	5.00	7.18	14.79	8.99	5.14
4	48.32	13.89	17.68	17.47	16.35	2.13
5	60.40	23.69	25.53	23.23	24.15	1.22

Table D5 Test of Homogeneity of Variances on the IC_{50} values of tyrosinase inhibition

Test of Homogeneity of Variances

ic50

Levene Statistic	df1	df2	Sig.
4.505	2	6	.064

Table D6 One-way analysis of variance on the IC$_{50}$ values of tyrosinase
inhibition

ANOVA

ic50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.615	2	.808	273.733	.000
Within Groups	.018	6	.003		
Total	1.633	8			

Table D7 Multiple Comparisons on the IC$_{50}$ values of tyrosinase inhibition by Tukey
method

Multiple Comparisons

Dependent Variable: ic50

Tukey HSD

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
emblica extract	gallic acid	.0875000	.0443471	.200	-.048569	.223569
	kojic acid	.9391667*	.0443471	.000	.803098	1.075236
gallic acid	emblica extract	-.0875000	.0443471	.200	-.223569	.048569
	kojic acid	.8516667*	.0443471	.000	.715598	.987736
kojic acid	emblica extract	-.9391667*	.0443471	.000	-1.075236	-.803098
	gallic acid	-.8516667*	.0443471	.000	-.987736	-.715598

*. The mean difference is significant at the .05 level.

Table D8 Homogeneous subset on the IC$_{50}$ values of tyrosinase inhibition by Tukey
method

ic50

Tukey HSD[a]

code	N	Subset for alpha = .05 1	2
kojic acid	3	.031667	
gallic acid	3		.883333
emblica extract	3		.970833
Sig.		1.000	.200

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.
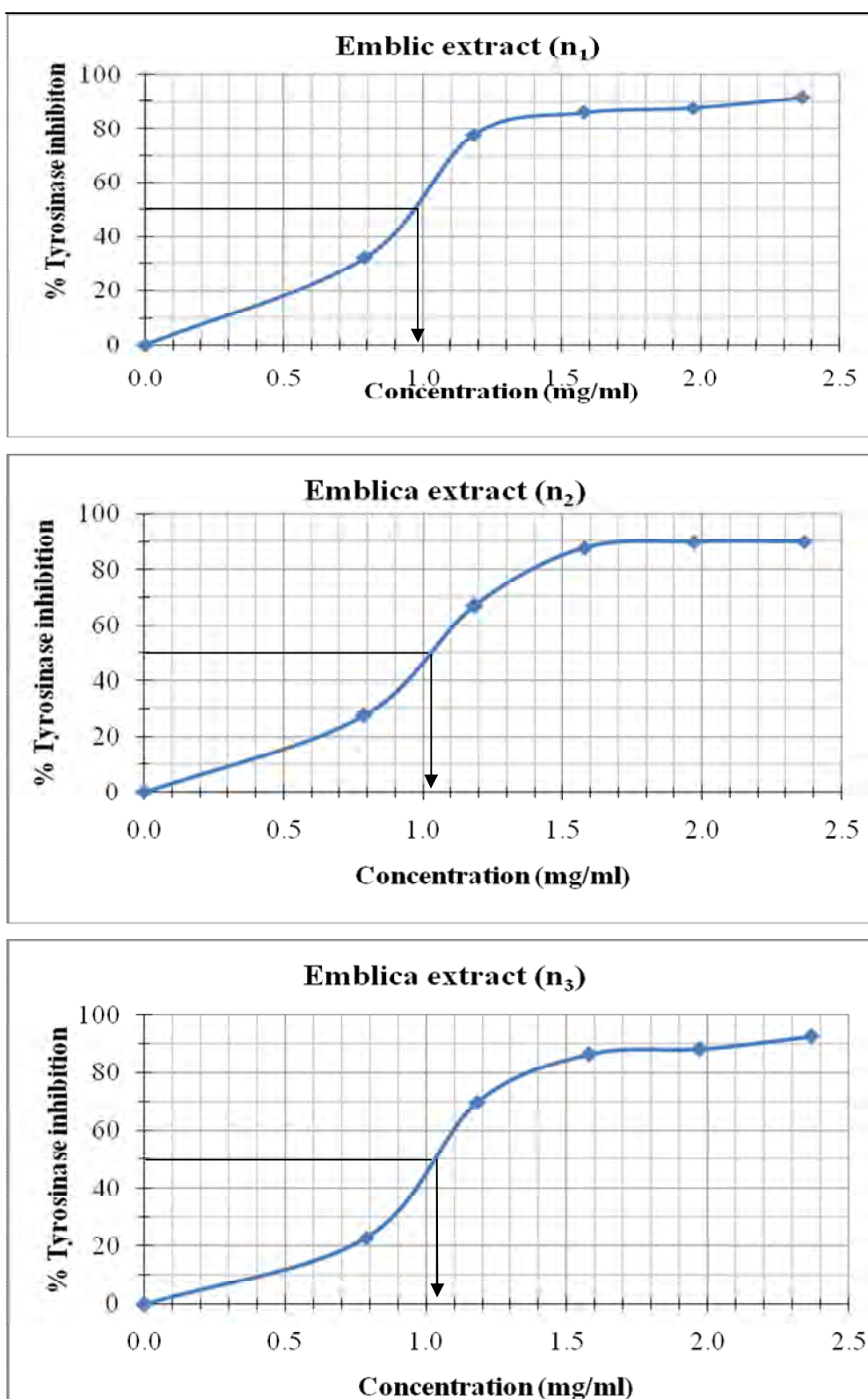
Figure D1 The relation of the % tyrosinase inhibition-concentration profile of emblica extract
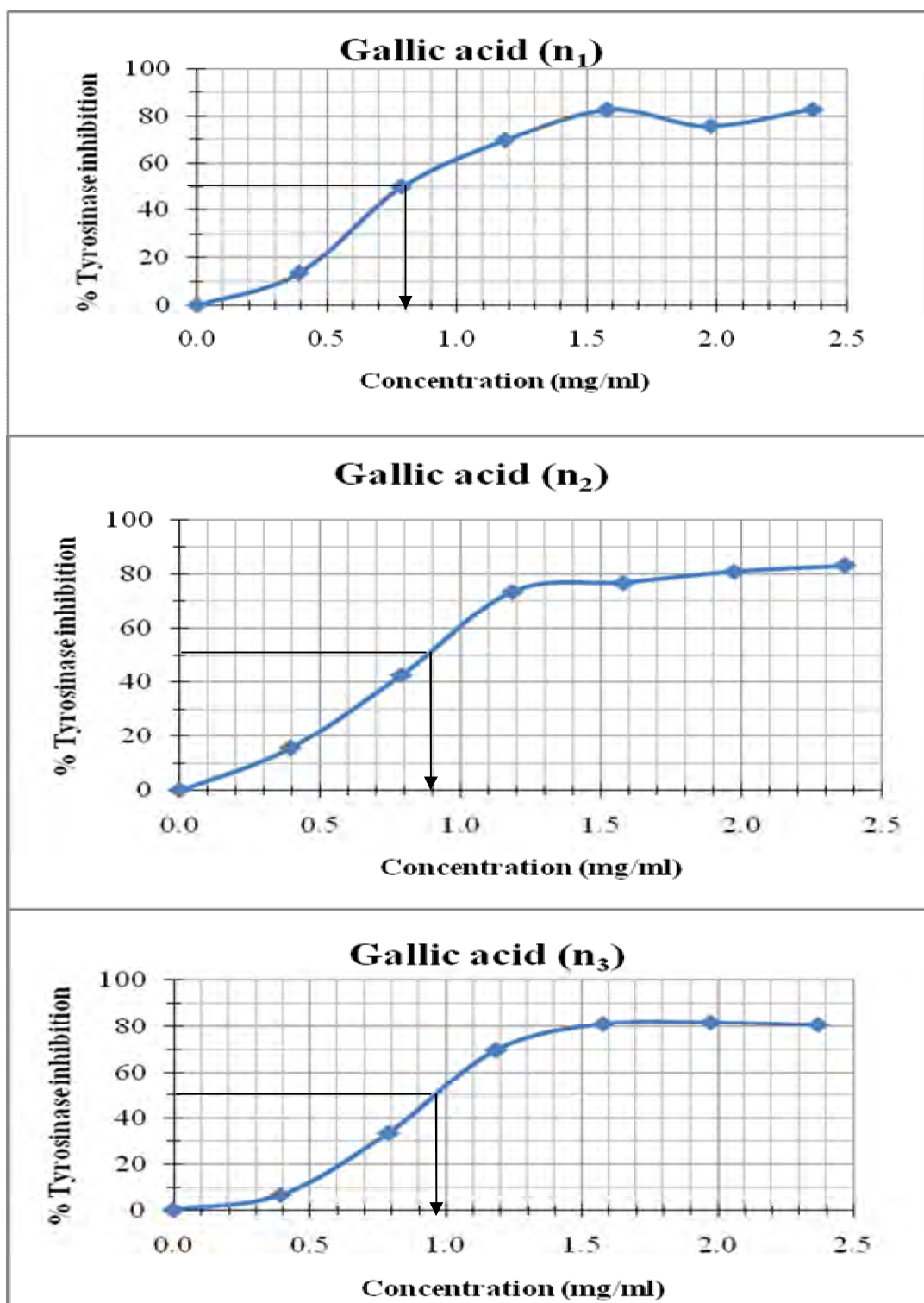
Figure D2 The relation of the % tyrosinase inhibition-concentration profile of standard gallic acid
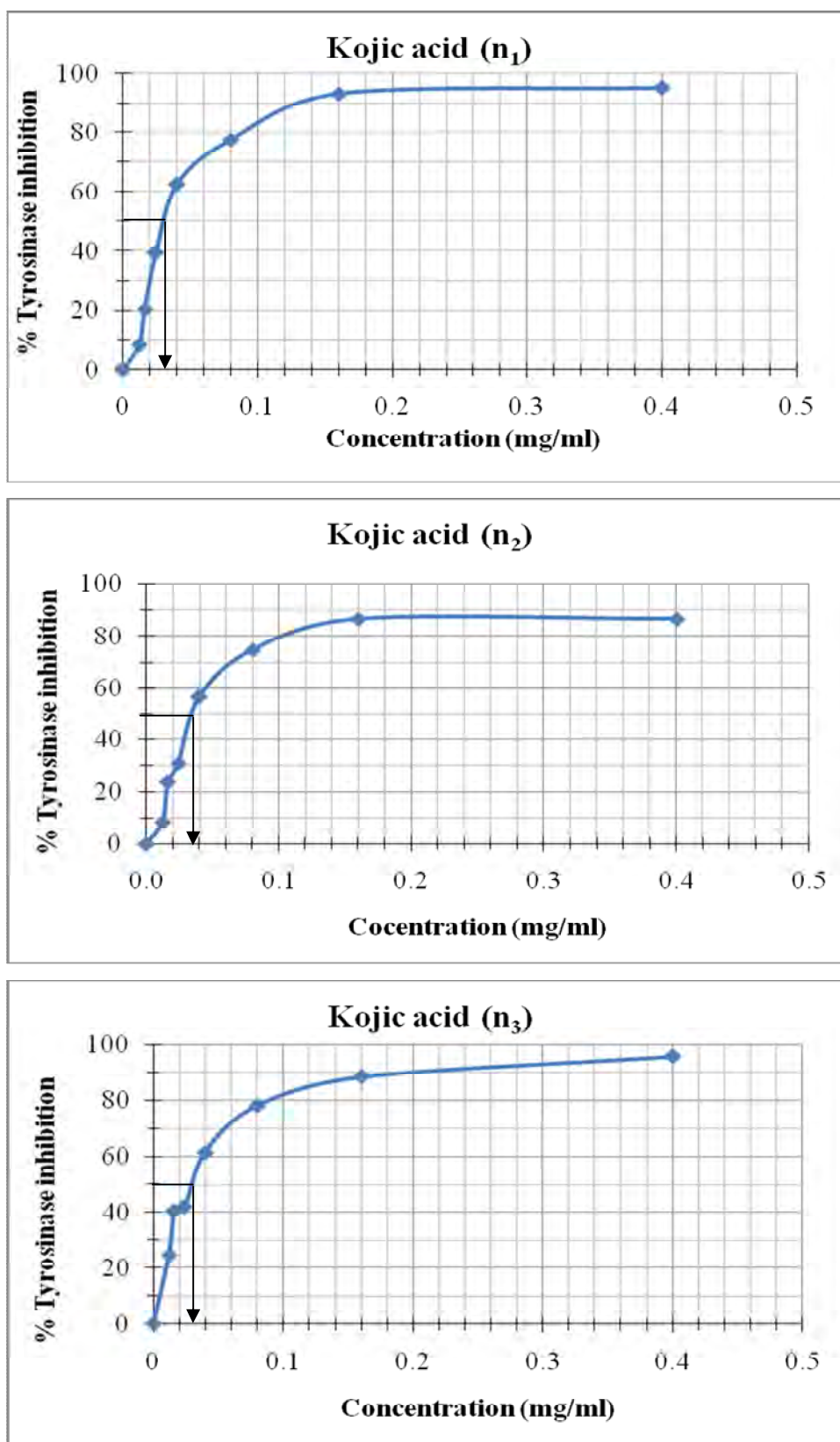
Figure D3 The relation of the % tyrosinase inhibition-concentration profile of kojic acid

APPENDIX E

Determination of Entrapment Efficiency

and Stability of Niosomes

Table E1 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at initial time under refrigerator

Formulaiton	% EE			Mean	SD
	n1	n2	n3		
N 20 11	41.06	34.58	42.20	39.28	4.11
N 20 64	44.03	52.51	51.34	49.29	4.60
N 40 11	38.13	36.75	39.72	38.20	1.49
N 40 64	38.69	37.02	35.20	36.97	1.75
N 40 73	33.74	36.39	32.93	34.35	1.81
N 60 11	39.68	37.83	37.30	38.27	1.25
N 60 64	43.52	38.94	41.88	41.45	2.32
N 60 73	38.18	36.18	34.32	36.23	1.93
N 80 64	39.09	43.43	41.26	41.26	2.17

Table E2 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at week2 under refrigerator

Formulation	% EE			Mean	SD
	n1	2 wk	n3		
N 20 11	40.29	34.38	42.01	37.52	4.00
N 20 64	40.90	49.94	45.32	43.66	4.52
N 40 11	32.61	36.19	35.23	35.31	1.85
N 40 64	38.28	36.73	34.85	34.58	1.72
N 40 73	34.45	36.14	32.85	30.47	1.65
N 60 11	39.39	37.14	36.20	37.58	1.64
N 60 64	41.37	38.23	39.90	37.59	1.57
N 60 73	38.00	36.65	34.36	37.10	1.84

Table E3 The data for entrapment efficiency study (% entrapment) of
niosomes dispersion at week4 under refrigerator

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	38.60	31.76	37.70	36.02	3.72
N 20 64	39.34	48.28	45.21	44.28	4.54
N 40 11	32.41	35.81	32.75	33.66	1.87
N 40 64	36.59	36.58	34.54	35.90	1.18
N 40 73	32.20	33.15	32.35	32.57	0.51
N 60 11	35.86	37.19	32.61	35.22	2.36
N 60 64	39.11	36.25	38.95	38.10	1.61
N 60 73	36.30	30.39	33.93	33.54	2.97

Table E4 The data for entrapment efficiency study (% entrapment) of
niosomes dispersion at week8 under refrigerator

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	36.61	31.51	35.58	34.57	2.70
N 20 64	39.13	46.59	43.49	43.07	3.75
N 40 11	31.07	35.09	32.98	33.05	2.01
N 40 64	35.46	35.57	33.67	34.90	1.07
N 40 73	32.02	31.52	32.03	31.86	0.29
N 60 11	30.41	36.20	32.60	33.07	2.92
N 60 64	37.24	35.36	37.66	36.75	1.22
N 60 73	35.30	30.37	33.89	33.19	2.54

Table E5 The data for entrapment efficiency study (% entrapment) of
niosomes dispersion at week12 under refrigerator

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	36.03	29.20	32.93	32.72	3.42
N 20 64	39.01	43.55	42.35	41.64	2.35
N 40 11	30.42	34.53	29.83	31.59	2.56
N 40 64	29.18	34.17	27.09	30.15	3.64
N 40 73	31.09	24.12	31.47	28.89	4.14
N 60 11	31.99	35.88	30.87	32.91	2.63
N 60 64	37.20	28.68	37.37	34.42	4.97
N 60 73	34.35	29.87	32.47	32.23	2.25

Table E6 The data for recovery study (% entrapment) of niosomes
dispersion at initial time under refrigerator

Formulation	% recovery			mean	SD
	n1	n2	n3		
N 20 11	97.69	97.30	96.62	97.20	0.54
N 20 64	100.07	100.40	101.94	100.80	1.00
N 40 11	95.27	98.33	96.23	96.61	1.56
N 40 64	98.83	99.29	99.16	99.09	0.24
N 40 73	97.16	95.87	100.38	97.80	2.32
N 60 11	98.79	97.31	97.83	97.98	0.75
N 60 64	100.78	100.20	99.62	100.20	0.58
N 60 73	98.77	97.23	99.52	98.51	1.17
N 80 64	101.71	93.17	98.28	97.72	4.30

Table E7 The data for recovery study (% entrapment) of niosomes
dispersion at week 2 under refrigerator

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	95.12	96.23	93.83	95.06	1.20
N 20 64	98.88	100.15	99.34	99.46	0.64
N 40 11	95.71	97.16	96.78	96.55	0.75
N 40 64	97.71	98.37	97.32	97.80	0.53
N 40 73	93.58	93.16	94.58	93.77	0.73
N 60 11	96.41	98.51	97.82	97.58	1.07
N 60 64	100.13	97.96	98.08	98.72	1.22
N 60 73	98.78	94.18	97.52	96.83	2.38

Table E8 The data for recovery study (% entrapment) of niosomes
dispersion at week 4 under refrigerator

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	94.53	93.89	93.94	94.12	0.36
N 20 64	96.78	95.95	98.94	97.22	1.54
N 40 11	96.89	96.42	96.12	96.48	0.39
N 40 64	98.20	97.71	95.51	97.14	1.43
N 40 73	94.72	91.68	93.03	93.14	1.52
N 60 11	94.59	96.26	96.07	95.64	0.91
N 60 64	96.89	97.81	96.85	97.18	0.54
N 60 73	97.05	94.07	98.75	96.62	2.37

Table E9 The data for recovery study (% entrapment) of niosomes dispersion at week 8 under refrigerator

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	95.37	93.63	94.76	94.59	0.88
N 20 64	91.72	94.63	94.96	93.77	1.78
N 40 11	92.18	95.05	86.28	91.17	4.47
N 40 64	94.95	96.30	94.77	95.34	0.84
N 40 73	90.90	91.06	93.18	91.71	1.27
N 60 11	92.61	93.21	95.96	93.93	1.79
N 60 64	97.79	95.66	95.90	96.45	1.17
N 60 73	94.88	93.46	96.42	94.92	1.48

Table E10 The data for recovery study (% entrapment) of niosomes dispersion at week 12 under refrigerator

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	89.54	85.92	89.13	88.20	1.98
N 20 64	88.46	94.39	96.79	93.21	4.29
N 40 11	89.96	92.10	85.57	89.21	3.33
N 40 64	92.09	96.76	87.02	91.96	4.87
N 40 73	89.19	90.99	92.04	90.74	1.44
N 60 11	89.19	86.95	91.83	89.32	2.44
N 60 64	95.80	94.79	91.60	94.06	2.19
N 60 73	92.78	93.02	92.47	92.76	0.28

Table E11 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at week2 under room temperature

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	40.29	35.11	39.20	38.20	2.73
N 20 64	40.90	49.83	52.14	47.62	5.93
N 40 11	32.41	41.47	34.10	35.99	4.81
N 40 64	38.28	37.32	36.35	37.32	0.96
N 40 73	34.45	32.99	31.27	32.90	1.59
N 60 11	39.39	36.53	36.30	37.41	1.72
N 60 64	39.37	37.82	39.65	38.95	0.98
N 60 73	31.02	36.37	34.55	33.98	2.72

Table E12 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at week 4 under room temperature

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	36.60	30.29	40.77	35.89	5.27
N 20 64	39.34	48.21	47.45	45.00	4.91
N 40 11	32.61	38.53	32.75	34.63	3.37
N 40 64	36.59	37.90	33.72	36.07	2.13
N 40 73	32.20	32.41	30.35	31.65	1.13
N 60 11	35.86	34.52	32.05	34.14	1.93
N 60 64	35.11	36.90	38.35	36.79	1.62
N 60 73	30.36	34.15	32.92	32.48	1.93

Table E13 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at week 8 under room temperature

Formulation	%EE			Mean	SD
	n1	n2	n3		
N 20 11	36.61	31.77	36.38	34.92	2.73
N 20 64	39.13	42.92	49.58	43.88	5.29
N 40 11	31.07	39.72	30.38	33.72	5.20
N 40 64	35.46	36.83	33.32	35.20	1.76
N 40 73	32.02	31.80	30.28	31.37	0.94
N 60 11	30.41	34.03	30.87	31.77	1.97
N 60 64	33.24	34.56	37.89	35.23	2.39
N 60 73	29.78	28.49	32.09	30.12	1.82

Table E14 The data for entrapment efficiency study (% entrapment) of niosomes dispersion at week 12 under room temperature

Formulation	% EE			Mean	SD
	n1	n2	n3		
N 20 11	36.03	30.97	32.78	33.26	2.56
N 20 64	39.01	41.61	41.32	40.65	1.42
N 40 11	30.42	34.11	26.25	30.26	3.93
N 40 64	29.18	33.22	28.80	30.40	2.44
N 40 73	31.09	24.79	28.06	27.98	3.15
N 60 11	31.99	31.67	29.50	31.05	1.35
N 60 64	30.20	27.66	37.38	31.75	5.04
N 60 73	29.50	28.19	32.50	30.06	2.20

Table E15 The data for recovery study (% entrapment) of niosomes dispersion at week 2 under room temperature

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	93.83	94.52	90.94	93.10	1.90
N 20 64	96.40	101.83	99.15	99.13	2.72
N 40 11	98.27	100.03	88.28	95.53	6.34
N 40 64	97.32	99.29	96.08	97.56	1.62
N 40 73	94.70	94.61	94.58	94.63	0.06
N 60 11	98.64	97.52	88.01	94.72	5.84
N 60 64	99.00	98.60	95.17	97.59	2.11
N 60 73	99.68	93.02	94.00	95.57	3.60

Table E16 The data for recovery study (% entrapment) of niosomes dispersion at week 4 under room temperature

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	90.53	93.89	87.85	90.76	3.03
N 20 64	91.27	97.68	98.58	95.84	3.99
N 40 11	94.24	94.21	82.33	90.26	6.87
N 40 64	99.82	94.71	95.51	96.68	2.75
N 40 73	90.19	95.35	91.47	92.34	2.69
N 60 11	94.42	95.34	86.89	92.22	4.64
N 60 64	90.75	98.70	96.85	95.43	4.16
N 60 73	96.81	94.90	96.41	96.04	1.01

Table E17 The data for recovery study (% entrapment) of niosomes dispersion at week 8 under room temperature

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	86.44	92.14	88.95	89.18	2.86
N 20 64	85.52	98.84	96.79	93.72	7.17
N 40 11	92.27	84.97	85.57	87.60	4.05
N 40 64	94.43	95.85	96.85	95.71	1.22
N 40 73	85.53	82.01	92.04	86.53	5.09
N 60 11	93.28	94.48	80.16	89.31	7.94
N 60 64	85.70	96.80	92.31	91.60	5.58
N 60 73	92.88	89.18	90.11	90.72	1.92

Table E18 The data for recovery study (% entrapment) of niosomes dispersion at week 12 under room temperature

Formulation	% recovery			Mean	SD
	n1	n2	n3		
N 20 11	79.51	71.99	81.63	77.71	5.07
N 20 64	81.97	75.98	90.07	82.67	7.07
N 40 11	91.81	85.34	80.08	85.74	5.88
N 40 64	86.80	94.44	89.46	90.23	3.88
N 40 73	81.26	80.43	89.25	83.65	4.87
N 60 11	91.00	79.54	76.46	82.33	7.66
N 60 64	81.18	80.06	91.60	84.28	6.36
N 60 73	93.82	73.67	86.39	84.63	10.19

Table E19 homogenity of variance on the percentages of entrapped gallic acid at initial time

Levene Statistic	df1	df2	Sig.
1.998	8	18	.106

Table E20 One-way analysis of variance on the percentages of entrapped gallic acid at initial time

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	509.402	8	63.675	8.858	.000
Within Groups	129.386	18	7.188		
Total	638.788	26			

Table E21 Homogeneous subset on the percentages of entrapped gallic acid at initial time

Tukey HSD

		Subset for alpha = .05	
code	N	1	2
n6073	3	33.8800	
n4073	3	34.3533	
n4064	3	36.9700	
n4011	3	38.2000	
n6011	3	38.2700	
n2011	3	39.2800	
n8064	3	41.2600	
n6064	3	41.4467	
n2064	3		49.2933
Sig.		.055	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Table E22 homogenity of variance on the percentages of entrapped gallic acid at initial time

Test of Homogeneity of Variances

EE

Levene Statistic	df1	df2	Sig.
1.932	15	32	.058

Table E23 One-way analysis of variance on the entrapment efficiency of gallic acid at initial time and week 12 under storage at 4 ℃

ANOVA

EE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1175.022	15	78.335	7.431	.000
Within Groups	337.347	32	10.542		
Total	1512.370	47			

Table E24 Homogeneous subset on the percentages of the entrapment efficiency of gallic acid at initial time and week 12 under storage at 4 ℃

EE

Tukey HSD[a]

code	N	Subset for alpha = .05			
		1	2	3	4
n4073t12	3	28.8933			
n4064t12	3	30.1467	30.1467		
n4011t12	3	31.6033	31.6033		
n2011t12	3	32.7200	32.7200	32.7200	
n6011t12	3	32.9133	32.9133	32.9133	
n4073t0	3	34.3533	34.3533	34.3533	
n6064t12	3	34.4167	34.4167	34.4167	
n6073t12	3	34.4167	34.4167	34.4167	
n6073t0	3	36.2267	36.2267	36.2267	
n4064t0	3	36.9700	36.9700	36.9700	
n4011t0	3	38.2000	38.2000	38.2000	
n6011t0	3	38.2700	38.2700	38.2700	
n2011t0	3		39.4600	39.4600	
n6064t0	3			41.4467	41.4467
n2064t12	3			41.6367	41.6367
n2064t0	3				49.2933
Sig.		.074	.078	.109	.241

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table E25 homogenity of variance on the percentages of entrapped gallic acid at initial time

Test of Homogeneity of Variances

EE

Levene Statistic	df1	df2	Sig.
1.638	15	32	.118

Table E26 One-way analysis of variance on the entrapment efficiency of gallic acid at initial time and week 12 under storage at room temperature (30 ºC)

ANOVA

EE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1394.709	15	92.981	11.412	.000
Within Groups	260.719	32	8.147		
Total	1655.428	47			

Table E27 Homogeneous subset on the percentages of the entrapment efficiency of gallic acid at initial time and week 12 under storage at room temperature (30 ºC)

EE

Tukey HSD[a]

		Subset for alpha = .05				
code	N	1	2	3	4	5
n4073t12	3	27.9800				
n6073t12	3	30.0633	30.0633			
n4011t12	3	30.2600	30.2600			
n4064t12	3	30.4000	30.4000			
n6011t12	3	31.0533	31.0533	31.0533		
n6064t12	3	31.7467	31.7467	31.7467		
n2011t12	3	33.2900	33.2900	33.2900	33.2900	
n4073t0	3	34.3533	34.3533	34.3533	34.3533	
n6073t0	3	36.2267	36.2267	36.2267	36.2267	
n4064t0	3		36.9700	36.9700	36.9700	
n4011t0	3		38.2000	38.2000	38.2000	
n6011t0	3		38.2700	38.2700	38.2700	
n2011t0	3			39.4600	39.4600	
n2064t12	3				40.6467	
n6064t0	3				41.4467	41.4467
n2064t0	3					49.2933
Sig.		.074	.077	.063	.081	.108

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table E28 homogenity of variance on the percentages loss of content of gallic acid at week 12 under storage condition at 4ºC

Test of Homogeneity of Variances

loss

Levene Statistic	df1	df2	Sig.
1.463	6	14	.260

Table E29 One-way analysis of variance the percentages loss of content of gallic acid at week 12 under storage condition at 4ºC

ANOVA

loss

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.438	6	5.073	.910	.516
Within Groups	78.062	14	5.576		
Total	108.500	20			

Table E30 homogenity of variance on the percentages loss of content of gallic acid at week 12 under storage condition at room temperature (30ºC)

Test of Homogeneity of Variances

loss

Levene Statistic	df1	df2	Sig.
.742	7	16	.641

Table E31 One-way analysis of variance the percentages loss of content of gallic acid at week 12 under storage condition at room temperature (30ºC)

ANOVA

loss

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	264.352	7	37.765	.916	.519
Within Groups	659.302	16	41.206		
Total	923.654	23			

APPENDIX F

Determination of Physicochemical

Properties and Stability of Niosome Serums

Table F5 Test of homogenity of variance of pH of NS6064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 4 ºC

Test of Homogeneity of Variances

ph

Levene Statistic	df1	df2	Sig.
2.320	4	10	.128

Table F6   One-way analysis of variance of pH of NS6064 serum under storage condition at 4 ºC

ANOVA

ph

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.025	4	.006	2.278	.133
Within Groups	.027	10	.003		
Total	.052	14			

Table F7 Test of homogenity of variance of pH of NS2064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 30 ºC

Test of Homogeneity of Variances

ph

Levene Statistic	df1	df2	Sig.
4.667	4	10	.022

Table F8 Multiple comparion of viscosity of NS2064 serum under storage condition at room temperature (30 ºC) by Dunett 3T test

Multiple Comparisons

Dependent Variable: ph
Dunnett T3

(I) formulation	(J) formulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
t0wk	t2wk	.03333	.03018	.910	-.1250	.1917
	t4wk	.03667	.04643	.978	-.2691	.3424
	t8wk	-.03667	.01764	.520	-.1574	.0841
	t12wk	-.04333	.04643	.951	-.3491	.2624
t2wk	t0wk	-.03333	.03018	.910	-.1917	.1250
	t4wk	.00333	.05011	1.000	-.2738	.2804
	t8wk	-.07000	.02582	.365	-.2678	.1278
	t12wk	-.07667	.05011	.730	-.3538	.2004
t4wk	t0wk	-.03667	.04643	.978	-.3424	.2691
	t2wk	-.00333	.05011	1.000	-.2804	.2738
	t8wk	-.07333	.04372	.679	-.4331	.2865
	t12wk	-.08000	.06128	.835	-.3752	.2152
t8wk	t0wk	.03667	.01764	.520	-.0841	.1574
	t2wk	.07000	.02582	.365	-.1278	.2678
	t4wk	.07333	.04372	.679	-.2865	.4331
	t12wk	-.00667	.04372	1.000	-.3665	.3531
t12wk	t0wk	.04333	.04643	.951	-.2624	.3491
	t2wk	.07667	.05011	.730	-.2004	.3538
	t4wk	.08000	.06128	.835	-.2152	.3752
	t8wk	.00667	.04372	1.000	-.3531	.3665

Table F9 Test of homogenity of variance of pH of NS4064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at room temperature (30 ºC)

Test of Homogeneity of Variances

ph

Levene Statistic	df1	df2	Sig.
2.785	4	10	.086

Table F10   One-way analysis of variance of pH of NS4064 serum under storage condition at room temperature (30 ºC)

ANOVA

ph

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.040	4	.010	9.018	.062
Within Groups	.011	10	.001		
Total	.051	14			

Table F11 Test of homogenity of variance of pH of NS6064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at room temperature (30 ºC)

Test of Homogeneity of Variances

ph

Levene Statistic	df1	df2	Sig.
1.953	4	10	.178

Table F12   One-way analysis of variance of pH of NS6064 serum under storage condition at room temperature (30 ºC)

ANOVA

ph

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.034	4	.009	2.680	.094
Within Groups	.032	10	.003		
Total	.066	14			

Table F13 Test of homogenity of variance of viscosity of NS2064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 4 ºC

Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
1.350	4	10	.318

Table F14 One-way analysis of variance of viscosity of NS2064 serum under storage condition at 4 ºC

ANOVA

viscosity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12208.000	4	3052.000	.822	.540
Within Groups	37143.333	10	3714.333		
Total	49351.333	14			

Table F 15 Test of homogenity of variance of viscosity of NS4064 serum at initial
time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 4 ºC

Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
5.257	4	10	.015

Table F16 Multiple comparion of viscosity of NS4064 serum under storage
condition at 4 ºC by Dunett 3T test

Multiple Comparisons

Dependent Variable: viscosity
Dunnett T3

(I) formulation	(J) formulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
t0wk	t2wk	-73.333	99.652	.982	-895.75	749.08
	t4wk	-69.000	99.249	.987	-903.42	765.42
	t8wk	-22.667	104.322	1.000	-743.00	697.67
	t12wk	-118.333	99.419	.866	-947.61	710.94
t2wk	t0wk	73.333	99.652	.982	-749.08	895.75
	t4wk	4.333	15.581	1.000	-75.43	84.10
	t8wk	50.667	35.715	.780	-185.49	286.82
	t12wk	-45.000	16.630	.276	-126.37	36.37
t4wk	t0wk	69.000	99.249	.987	-765.42	903.42
	t2wk	-4.333	15.581	1.000	-84.10	75.43
	t8wk	46.333	34.575	.811	-208.82	301.49
	t12wk	-49.333	14.016	.139	-117.97	19.30
t8wk	t0wk	22.667	104.322	1.000	-697.67	743.00
	t2wk	-50.667	35.715	.780	-286.82	185.49
	t4wk	-46.333	34.575	.811	-301.49	208.82
	t12wk	-95.667	35.060	.346	-341.74	150.41
t12wk	t0wk	118.333	99.419	.866	-710.94	947.61
	t2wk	45.000	16.630	.276	-36.37	126.37
	t4wk	49.333	14.016	.139	-19.30	117.97
	t8wk	95.667	35.060	.346	-150.41	341.74

Table F17 Test of homogenity of variance of viscosity of NS6064 serum at initial
time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 4 ºC
Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
.740	4	10	.586

Table F18 One-way analysis of variance of viscosity of NS6064 serum under storage condition at 4 ºC

ANOVA

viscosity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	77534.267	4	19383.567	2.354	.124
Within Groups	82354.667	10	8235.467		
Total	159888.9	14			

Table F19 Test of homogenity of variance of viscosity of NS2064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 30 ºC

Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
1.790	4	10	.207

Table F20 One-way analysis of variance of viscosity of NS2064 serum under storage condition at room temperature (30 ºC)

ANOVA

viscosity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1889.600	4	472.400	.142	.963
Within Groups	33265.333	10	3326.533		
Total	35154.933	14			

Table F 21 Test of homogenity of variance of viscosity of NS4064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at room temperature (30 ºC)

Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
1.415	4	10	.298

Table F22 One-way analysis of variance of viscosity of NS4064 serum under storage condition at room temperature (30 ºC)

ANOVA

viscosity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	171876.9	4	42969.233	2.348	.125
Within Groups	182970.0	10	18297.000		
Total	354846.9	14			

Table F23 Test of homogenity of variance of viscosity of NS6064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 30 ºC

Test of Homogeneity of Variances

viscosity

Levene Statistic	df1	df2	Sig.
1.724	4	10	.221

Table F24 One-way analysis of variance of viscosity of NS6064 serum under storage condition at room temperature (30 ºC)

ANOVA

viscosity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	255718.667	4	63929.667	4.043	.053
Within Groups	158117.333	10	15811.733		
Total	413836.000	14			

Table F25 Percentage contents of gallic acid remaining after 12 weeks at 4 ºC

Formulation	Gallic acid content (%)			
	week 2	week 4	week 8	week 12
NS206401				
1	98.38	94.32	90.35	78.08
2	96.32	93.84	92.45	89.19
3	98.46	90.73	92.90	80.07
Mean±SD	97.72±1.21	92.96±1.95	91.90±1.36	82.45±5.92
NS406401				
1	99.34	84.84	82.53	79.10
2	96.81	92.81	89.37	75.20
3	96.08	93.38	82.99	77.54
Mean±SD	97.41±1.71	90.34±4.77	84.96±3.82	77.28±1.96
NS606401				
1	98.97	96.96	90.84	79.06
2	85.17	83.10	79.09	77.58
3	90.22	88.17	81.15	81.41
Mean±SD	91.45±6.98	89.41±7.01	83.69±6.28	79.35±1.93

Table F26 Percentage contents of gallic acid remaining after 12 weeks at room temperature (30 ºC)

Formulation	Gallic acid content (%)			
	week 2	week 4	week 8	week 12
NS206401				
1	86.39	73.55	77.44	55.59
2	94.64	102.26	86.43	67.28
3	84.90	87.53	79.49	69.40
Mean±SD	88.64±5.25	87.78±14.36	81.12±4.71	64.09±7.44
NS406401				
1	100.90	88.93	84.35	72.42
2	81.86	75.31	76.85	71.19
3	78.03	72.24	71.39	69.84
Mean±SD	86.93±12.25	78.83±8.88	77.53±6.50	71.15±1.29
NS606401				
1	85.88	86.21	82.76	68.53
2	86.03	78.80	73.61	60.14
3	99.42	92.71	86.50	72.59
Mean±SD	90.44±7.77	85.91±6.96	80.95±6.63	67.09±6.35

Table F 27 Test of homogenity of variance of the percentage of gallic acid of NS2064 serum at initial time, t 2 wk, t 4 wk , t 8 wk and t 12 wk under storage condition at 4 °C

Test of Homogeneity of Variances

content

Levene Statistic	df1	df2	Sig.
4.086	14	30	.001

Table F28 Multiple comparion of the percentage of gallic acid of NS2064 serum

under storage condition at 4 °C by Dunett 3T test

Multiple Comparisons

Post Hoc Tests

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ns20 t0	ns20 t2	2 28000	.70038	.510	-6 3431	10.9031
	ns20t4	7 03667	1.12623	179	-6 8172	20.8905
	ns20t8	8 10000	.78581	070	-1.5749	17.7749
	ns20t12	17.55333	3.42026	253	-24.5568	59.6635
	ns40t0	.00000	.00000	.	.0000	.0000
	ns40t2	2.59000	98774	661	-9.5711	14.7511
	ns40t4	9.65667	2.75658	461	-24.2824	43.5957
	ns40t8	15.03667	2.20733	154	-12.1400	42.2133
	ns40t12	22.72000*	1.13331	.019	8.7666	36.6734
	ns60t0	.00000	00000		.0000	.0000
	ns60t2	8.54667	4 03116	802	-41.0850	58.1783
	ns60t4	10.59000	4 04879	.663	39.2587	60.4387
	ns60t8	16.30667	3 62248	315	-28.2933	60 9066
	ns60t12	20 65000*	1 11509	.022	6.9210	34.3790
ns20 t2	ns20 t0	2.28000	.70038	.510	-10.9031	6.3431
	ns20t4	4.75667	1 32540	.364	-5.2466	14.7599
	ns20t8	5.82000	1 05263	.098	-1.2988	12.9388
	ns20t12	15 27333	3.49123	.314	-23 8230	54 3696
	ns40t0	-2 28000	.70038	.510	-10.9031	6 3431
	ns40t2	31000	1.21085	1.000	-8.3694	8 9894
	ns40t4	7.37667	2.84417	.664	-23 0773	37.8306
	ns40t8	12.75667	2.31578	.187	-10 5089	36 0222
	ns40t12	20.44000*	1.33227	.005	10 3539	30 5261
	ns60t0	-2 28000	.70038	510	-10 9031	6.3431
	ns60t2	6.26667	4.09155	.951	-40 7095	53 2428
	ns60t4	8.31000	4.10892	.832	-33.8925	55 5125
	ns60t8	14.02667	3.68956	.397	-27 6860	55 7393
	ns60t12	18.37000*	1.31680	.007	3 4699	28 2701

Table F28 Multiple comparion of the percentage of gallic acid of NS2064 serum
under storage condition at 4 ºC by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) ccde	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ns20t4	ns20 t0	-7.03667	1 12523	.179	-20.8905	6.8172
	ns20 t2	-4.75667	1 32540	.364	-14.7599	5.2466
	ns20t8	.06333	1 37246	1.000	-8.8287	10.9554
	ns20t12	10.51667	3 60060	.568	-25.2174	46.2508
	ns40t0	-7.03667	1 12523	.179	-20.8905	6.8172
	ns40t2	-4.44667	1.49726	512	-14.5948	5.7015
	ns40t4	2.62000	2.97740	1 000	-24.5682	29.8082
	ns40t8	8.00000	2 47759	466	-12.4734	28 4734
	ns40t12	15.68333*	1.59704	013	4 9679	26.3988
	ns60t0	-7.03667	1.12523	.179	-20 8905	6.8172
	ns60t2	1.51000	4.18526	1.000	-42 2133	45.2333
	ns60t4	3 55333	4.20224	1.000	40 4008	47.5075
	ns60t8	9.27000	3.79322	.707	-29.1038	47.6438
	ns60t12	13.61333*	1.58416	.021	2 9841	24.2425
ns20t8	ns20 t0	-8.10000	.78581	.070	-17.7749	1 5749
	ns20 t2	-5.82000	1.05263	.098	-12.9388	1 2988
	ns20t4	-1.06333	1.37246	1.000	-10.9554	8 8287
	ns20t12	9.45333	3.50937	.637	-28.9903	47.8970
	ns40t0	-8.10000	.78581	.070	-17.7749	1 5749
	ns40t2	-5.51000	1 26219	.208	-14.2411	3 2211
	ns40t4	1.55667	2 86640	1 000	-28.2102	31 3236
	ns40t8	6.93667	2 34303	555	-15.6646	29 5380
	ns40t12	14.62000*	1 37909	.014	4.6539	24.5861
	ns60t0	-8.10000	78581	.070	-17.7749	1.5749
	ns60t2	.44667	4 10704	1 000	-45.9253	46.8187
	ns60t4	2.49000	4.12434	1.000	-44.1098	49.0898
	ns60t3	8.20667	3.70673	.777	-32.8687	49.2821
	ns60t12	12.55000*	1.36416	.022	2.7500	22.3500
ns20t12	ns20 t0	17 55333	3.42026	.253	-59.6635	24.5568
	ns20 t2	-15 27333	3.49123	.314	-54.3696	23.8230
	ns20t4	-10 51667	3.60060	.568	-46.2508	25.2174
	ns20t8	-9 45333	3.50937	.637	-47.8970	28.9903
	ns40t0	-17 55333	3.42026	.253	59 6635	24.5568
	ns40t2	-14.96333	3.56002	.322	-51 7942	21.8676
	ns40t4	-7.89667	4 39282	.923	-38 1846	22.3913
	ns40t3	-2.51667	4 07068	1 000	-32 7751	27.7417
	ns40t12	5.16667	3 60313	.972	-30 5040	40 8374
	ns60t0	-17.55333	3 42026	.253	59 6635	24.5568
	ns60t2	-9.00667	5 28663	.946	45 0499	27.0365
	ns60t4	-6.96333	5 30008	.994	-43 1289	29.2022
	ns60t8	-1.24667	4 98202	1.000	-34.7393	32.2460
	ns60t12	3.09667	3 59744	1.000	-32.7172	38.9105

Table F28 Multiple comparion of the percentage of gallic acid of NS2064 serum under storage condition at 4 ℃ by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dumett T3

(I) code	(J) code	Mean Difference (I-J)	Std Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
ns40t0	ns20 t0	.00000	.00000		.0000	.0000
	ns20 t2	2.28000	.70038	.510	-6.3431	10.9031
	ns20t4	7.03667	1.12523	.179	-6.8172	20.8905
	ns20t8	8.10000	.78581	.070	-1.5749	17.7749
	ns20t12	17.55333	3.42026	.253	-24.5568	59.6635
	ns40t2	2.59000	.98774	.661	-9.5711	14.7511
	ns40t4	9.65667	2.75658	.461	-24.2824	43.5957
	ns40t8	15.03667	2.20733	.154	-12.1400	42.2133
	ns40t12	22.72000*	1.13331	.019	3.7666	36.6734
	ns60t0	.00000	.00000	.	.0000	.0000
	ns60t2	8.54667	4.03116	.802	-41.0850	58.1783
	ns60t4	10.59000	4.04879	.663	39.2587	60.4387
	ns60t8	16.30667	3.62248	.315	-28.2933	60.9066
	ns60t12	20.65000*	1.11509	.022	6.9210	34.3790
ns40t2	ns20 t0	-2.59000	.98774	.661	-14.7511	9.5711
	ns20 t2	-.31000	1.21085	1.000	-8.9894	8.3694
	ns20t4	4.44667	1.49726	.512	-5.7015	14.5948
	ns20t8	5.51000	1.26219	.208	-3.2211	14.2411
	ns20t12	14.96333	3.56002	.322	-21.8676	51.7942
	ns40t0	-2.59000	.98774	.661	-14.7511	9.5711
	ns40t4	7.06667	2.92820	.715	-21.1102	35.2435
	ns40t8	12.44667	2.41825	.183	-8.7671	33.6604
	ns40t12	20.13000*	1.50334	.004	9.9291	30.3309
	ns60t0	-2.59000	.98774	.661	-14.7511	9.5711
	ns60t2	5.95667	4.15041	.969	-38.8690	50.7824
	ns60t4	8.00000	4.16753	.863	-37.0559	53.0559
	ns60t8	13.71667	3.75473	.412	-25.7626	53.1959
	ns60t12	18.06000*	1.48965	.006	7.9770	28.1430
ns40t4	ns20 t0	-9.65667	2.75658	.461	-43.5957	24.2824
	ns20 t2	7.37667	2.84417	.664	-37.8306	23.0773
	ns20t4	-2.62000	2.97740	1.000	-29.8082	24.5682
	ns20t8	-1.55667	2.86640	1.000	-31.3236	28.2102
	ns20t12	7.89667	4.39282	.923	-22.3913	38.1846
	ns40t0	-9.65667	2.75658	.461	-43.5957	24.2824
	ns40t2	-7.06667	2.92820	.716	-35.2435	21.1102
	ns40t8	5.38000	3.53144	.975	-19.0080	29.7680
	ns40t12	13.06333	2.98046	.273	-14.0702	40.1969
	ns60t0	-9.65667	2.75658	.461	-43.5957	24.2824
	ns60t2	-1.11000	4.88355	1.000	-36.5859	34.3659
	ns60t4	.93333	4.89811	1.000	-34.7076	36.5743
	ns60t8	6.65000	4.55204	.982	-25.2311	38.5311
	ns60t12	10.99333	2.97358	.381	-16.2641	38.2507

Table F28 Multiple comparion of the percentage of gallic acid of NS2064 serum under storage condition at 4 °C by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
ns40t8	ns20 t0	-15.03667	2.20733	.154	-42.2133	12.1400
	ns20 t2	-12.75667	2.31578	.187	-36.0222	10.5089
	ns20t4	-3.00000	2.47759	.466	-28.4734	12.4734
	ns20t8	-6.93667	2.34303	.555	-29.5380	15.6646
	ns20:12	2.51667	4.07068	1.000	-27.7417	32.7751
	ns40t0	-15.03667	2.20733	.154	-42.2133	12.1400
	ns40t2	-12.44667	2.41825	.186	-33.6604	8.7671
	ns40t4	-5.38000	3.53144	.975	-29.7680	19.0080
	ns40t12	7.68333	2.48127	.500	-12.7524	28.1190
	ns60t0	-15.03667	2.20733	.154	-42.2133	12.1400
	ns60t2	-6.49000	4.59593	.982	43.2285	30.2485
	ns60t4	-4.44667	4.61140	1.000	41.3832	32.4899
	ns60t8	1.27000	4.24201	1.000	-31.0421	33.5821
	ns60t12	5.61333	2.47300	.763	-14.9082	26.1349
ns40t12	ns20 t0	22.72000*	1.13331	.019	-36.6734	-8.7666
	ns20 t2	-20.44000*	1.33227	.005	-30.5261	10.3539
	ns20t4	-15.68333*	1.59704	.013	-26.3988	-4.9679
	ns20t8	-14.62000*	1.37909	.014	-24.5861	-4.6539
	ns20t12	-5.16667	3.60313	.972	-40.8374	30.5040
	ns40t0	-22.72000*	1.13331	.019	-36.6734	-8.7666
	ns40t2	-20.13000*	1.50334	.004	-30.3309	-9.9291
	ns40t4	-13.06333	2.98046	.273	-40.1969	14.0702
	ns40t8	-7.68333	2.48127	.500	-28.1190	12.7524
	ns60t0	-22.72000*	1.13331	.019	-36.6734	-8.7666
	ns60t2	-14.17333	4.18744	.463	-57.8315	29.4848
	ns60t4	-12.13000	4.20441	.582	-56.0190	31.7590
	ns60t8	-6.41333	3.79562	.924	-44.7227	31.8961
	ns60t12	-2.07000	1.58992	.995	-12.7390	8.5990
ns60t0	ns20 t0	.00000	.00000		.0000	.0000
	ns20 t2	2.28000	.70038	.510	-6.3431	10.9031
	ns20t4	7.03667	1.12523	.179	-6.8172	20.8905
	ns20t8	8.10000	.78581	.070	-1.5749	17.7749
	ns20t12	17.55333	3.42026	.253	-24.5558	59.6635
	ns40t0	.00000	.00000		.0000	.0000
	ns40t2	2.59000	.98774	.661	-9.5711	14.7511
	ns40t4	9.65667	2.75658	.461	24.2824	43.5957
	ns40t8	15.03667	2.20733	.154	-12.1400	42.2133
	ns40t12	22.72000*	1.13331	.019	8.7666	36.6734
	ns60t2	8.54667	4.03116	.802	41.0850	58.1783
	ns60t4	10.59000	4.04879	.663	39.2587	60.4387
	ns60t8	16.30667	3.62248	.315	28.2933	60.9066
	ns60t12	20.65000*	1.11509	.022	6.9210	34.3790

Table F28 Multiple comparion of the percentage of gallic acid of NS2064 serum under storage condition at 4 ºC by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
ns60t2	ns20 t0	-8.54667	4.0316	.802	-58.1783	41.0850
	ns20 t2	-6.26667	4.09155	.951	-53.2428	40.7095
	ns20t4	-1.51000	4.18526	1.000	-45.2333	42.2133
	ns20t8	-.44667	4.10704	1.000	-46.8187	45.9253
	ns20t12	9.00667	5.28663	.946	-27.0365	45.0499
	ns40t0	-8.54667	4.03116	.802	-58.1783	41.0850
	ns40t2	-5.95667	4.15041	.969	-50.7824	38.8690
	rs40t4	1.11000	4.88355	1.000	-34.3659	36.5859
	rs40t8	6.49000	4.59593	.982	-30.2485	43.2285
	rs40t12	14.17333	4.18744	.463	-29.4848	57.8315
	ns60t0	-8.54667	4.03116	.802	-58.1783	41.0850
	ns60t4	2.04333	5.71340	1.000	-36.2902	40.3769
	ns60t8	7.76000	5.41965	.986	-28.8525	44.3725
	ns60t12	12.10333	4.18255	.580	-31.7017	55.9083
ns60t4	ns20 t0	-10.59000	4.04879	.663	50.4387	39.2587
	ns20 t2	-8.31000	4.10892	.832	55.5125	38.8925
	ns20t4	-3.55333	4.20224	1.000	47.5075	40.4003
	ns20t8	-2.49000	4.12434	1.000	-49.0898	44.1098
	ns20t12	6.96333	5.30008	.994	-29.2022	43.1289
	ns40t0	-10.59000	4.04879	.663	-60.4387	39.2587
	ns40t2	-8.00000	4.16753	.833	-53.0559	37.0559
	ns40t4	-.93333	4.8981	1.000	-36.5743	34.7076
	ns40t8	4.44667	4.61140	1.000	-32.4899	41.3832
	ns40t12	12.13000	4.2044	.582	-31.7590	56.0190
	ns60t0	-10.59000	4.04879	.663	-60.4387	39.2587
	ns60t2	-2.04333	5.71340	1.000	-40.3769	36.2902
	ns60t8	5.71667	5.43278	1.000	-31.0052	42.4386
	ns60t12	10.06000	4.19954	.722	-33.9759	54.0959
ns60t8	ns20 t0	-16.30667	3.62248	.315	-60.9066	28.2933
	ns20 t2	-14.02667	3.68956	.397	-55.7393	27.6860
	ns20t4	-9.27000	3.79322	.707	-47.6438	29.1038
	ns20t8	-8.20667	3.70673	.777	-49.2821	32.8687
	ns20t12	1.24667	4.98202	1.000	-32.2460	34.7393
	ns40t0	-16.30667	3.62248	.315	-60.9066	28.2933
	ns40t2	-13.71667	3.75473	.412	-53.1959	25.7626
	ns40t4	-6.65000	4.55204	.982	38.5311	25.2311
	ns40t8	-1.27000	4.24201	1.000	-33.5821	31.0421
	ns40t12	6.41333	3.79562	.924	31.8961	44.7227
	ns60t0	-16.30667	3.62248	.315	60.9066	28.2933
	ns60t2	-7.76000	5.41965	.986	-44.3725	28.8525
	ns60t4	-5.71667	5.43278	1.000	-42.4386	31.0052
	ns60t12	4.34333	3.79022	.995	-34.1115	42.7981

Table F29 Test of homogenity of variance of the percentage of gallic acid under
storage condition at room temperature (30 ºC)

Test of Homogeneity of Variances

content

Levene Statistic	df1	df2	Sig.
2.661	14	30	.012

Table F30 Multiple comparion of the percentage of gallic acid under storage

condition at room temperature (30ºC) by Dunett 3T test

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interva	
					Lower Bound	Upper Bound
ns20 t0	ns20 t2	11.35667	3 02903	.418	-25 9367	48 6501
	ns20t4	12.22000	8 28881	.958	-89.8318	114 2718
	ns20t8	18.88000	2 72015	.148	-14.6105	52.3705
	ns20t12	35 91000	4 29384	.105	-16.9557	88 7757
	ns40t0	.00000	00000		0000	0000
	ns40t2	13.07000	7 07196	.877	-74.0000	100 1400
	ns40t4	21 17333	5.12882	.361	-41.9726	84.3193
	ns40t8	22 47000	3.75665	194	-23.7818	68.7218
	ns40t12	28 85000*	74505	005	19.6769	38.0231
	ns60t0	00000	.00000		.0000	0000
	ns60t2	9 55667	4.48854	800	-45.7063	64.8196
	ns60t4	14 09333	4.01833	460	-35.3804	63.5671
	ns60t8	19 04333	3.82871	267	-28 0957	66 1824
	ns60t12	32 91333	3.66574	092	-12.2193	78.0460
ns20 t2	ns20 t0	-11 35667	3.02903	.418	-48.6501	25.9367
	ns20t4	.86333	8.82493	1 000	-83.4195	85.1462
	ns20t8	7.52333	4 07115	.912	-19 9816	35.0283
	ns20t12	24.55333	5.25472	.182	-13 1818	62.2885
	ns40t0	-11.35667	3.02903	.418	-48 6501	25.9367
	ns40t2	1.71333	7 69335	1.000	-67 0974	70.5240
	ns40t4	9.81667	5 95649	.948	-36 1862	55.8195
	ns40t8	11.11333	4 82570	.757	-22 1557	44 3824
	ns40t12	17.49333	3 11931	.194	16.1661	51.1528
	ns60t0	-11 35667	3.02903	.418	48.6501	25.9367
	ns60t2	-1.80000	5 41498	1.000	-41 3368	37 7368
	ns60t4	2.73667	5.03210	1.000	-32.6079	38 0812
	ns60t8	7 68667	4.88201	.968	-26.1334	41 5067
	ns60t12	21 55667	4.75528	.184	-11.0409	54.1542

Table F30 Multiple comparion of the percentage of gallic acid under storage
condition at room temperature (30ºC) by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnet T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
ns20t4	ns20t0	12.22000	8.28881	.958	-114.2718	89.8318
	ns20t2	-.86333	8.82493	1.000	85.1462	83.4195
	ns20t8	6.66000	8.72373	1.000	79.9930	93.3130
	ns20t12	23.69000	9.33495	.672	52.8746	100.2546
	ns40t0	-12.22000	8.28881	.958	114.2718	89.8318
	ns40t2	.85000	10.89573	1.000	73.3570	75.0570
	ns40t4	8.95333	9.74726	1.000	64.8002	82.7069
	ns40t8	10.25000	9.10037	.997	69.1255	89.6255
	ns40t12	16.63000	8.32222	.837	-83.8489	117.1089
	ns60t0	-12.22000	8.28881	.958	-114.2718	89.8318
	ns60t2	-2.66333	9.42610	1.000	-78.4006	73.0739
	ns60t4	1.87333	9.21148	1.000	-76.0380	79.7847
	ns60t8	6.82333	9.13035	1.000	-72.1316	85.7782
	ns60t12	20.69333	9.06322	.758	-59.2311	100.6178
ns20t8	ns20t0	-18.88000	2.72015	.148	-52.3705	14.6105
	ns20t2	-7.52333	4.07115	.912	-35.0283	19.9816
	ns20t4	-6.66000	8.72373	1.000	-93.3130	79.9930
	ns20t12	17.03000	5.08294	.418	-21.0464	55.1064
	ns40t0	18.88000	2.72015	.148	-52.3705	14.6105
	ns40t2	-5.81000	7.57706	1.000	-76.7254	65.1054
	ns40t4	2.29333	5.80551	1.000	-44.8052	49.3918
	ns40t8	3.59000	4.63806	1.000	-29.4141	36.5941
	ns40t12	9.97000	2.82034	.434	-19.6484	39.5884
	ns60t0	-18.88000	2.72015	.148	-52.3705	14.6105
	ns60t2	-9.32333	5.24845	.921	-49.3974	30.7508
	ns60t4	-4.78667	4.85245	1.000	-40.1736	30.6003
	ns60t8	.16333	4.69662	1.000	33.4787	33.8054
	ns60t12	14.03333	4.56475	.485	-18.1872	46.2539
ns20t12	ns20t0	-35.91000	4.29384	.105	-88.7757	16.9557
	ns20t2	-24.55333	5.25472	.182	-62.2885	13.1818
	ns20t4	-23.69000	9.33495	.672	-100.2546	52.8746
	ns20t8	-17.03000	5.08294	.418	-55.1064	21.0464
	ns40t0	-35.91000	4.29384	.105	-88.7757	16.9557
	ns40t2	-22.84000	8.27343	.594	-85.9589	40.2789
	ns40t4	-14.73667	6.68893	.794	-60.4617	30.9884
	ns40t8	-13.44000	5.70521	.737	-52.1289	25.2489
	ns40t12	7.06000	4.35800	.934	-57.1037	42.9837
	ns60t0	-35.91000	4.29384	.105	-88.7757	16.9557
	ns60t2	-26.35333	6.21161	.216	-68.0786	15.3719
	ns60t4	-21.81667	5.88082	.310	-61.3778	17.7445
	ns60t8	-16.86667	5.75292	.524	-55.7702	22.0369
	ns60t12	-2.99667	5.64577	1.000	-41.4436	35.4503

Table F30 Multiple comparion of the percentage of gallic acid under storage
condition at room temperature (30ºC) by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
ns40t0	ns20 t0	.00000	.00000		.0000	.0000
	ns20 t2	11.35667	3.02903	.418	-25.9367	48.6501
	rs20t4	12.22000	8.28881	.958	-89.8318	114.2718
	rs20t8	18.88000	2.72015	.148	-14.6105	52.3705
	rs20t12	35.91000	4.29384	.105	-16.9557	88.7757
	rs40t2	13.07000	7.07196	.877	-74.0000	100.1400
	rs40t4	21.17333	5.12882	.361	-41.9726	84.3193
	rs40t8	22.47000	3.75665	.94	-23.7818	68.7218
	rs40t12	28.85000	.74505	.005	19.6769	38.0231
	rs60t0	.00000	.00000	.	.0000	.0000
	rs60t2	9.55667	4.48854	.800	-45.7063	64.8196
	rs60t4	14.09333	4.01833	.460	-35.3804	63.5671
	rs60t8	19.04333	3.82871	.267	-28.0957	66.1824
	rs60t12	32.91333	3.66574	.092	-12.2193	78.0460
ns40t2	rs20 t0	-13.07000	7.07196	.877	-100.1400	74.0000
	ns20 t2	-1.71333	7.69335	1.000	-70.5240	67.0974
	ns20t4	-.85000	10.89573	1.000	-75.0570	73.3570
	ns20t8	5.81000	7.57706	1.000	-65.1054	76.7264
	ns20t12	22.84000	8.27343	.594	-40.2789	85.9589
	ns40t0	-13.07000	7.07196	.877	-100.1400	74.0000
	ns40t4	8.10333	8.73598	1.000	-54.0280	70.2347
	ns40t8	9.40000	8.00781	.997	-55.5176	74.3176
	ns40t12	15.78000	7.11110	.774	-69.4652	101.0252
	ns60t0	-13.07000	7.07196	.877	-100.1400	74.0000
	ns60t2	-3.51333	8.37614	1.000	-66.2094	59.1828
	ns60t4	1.02333	8.13386	1.000	-62.9014	64.9481
	ns60t8	5.97333	8.04187	1.000	-58.6488	70.5954
	ns60t12	19.84333	7.96557	.688	-45.4709	85.1576
ns40t4	ns20 t0	-21.17333	5.12862	.361	-84.3193	41.9726
	ns20 t2	-9.81667	5.95649	.948	-55.8195	36.1862
	ns20t4	-8.95333	9.74726	1.000	-82.7069	64.8002
	ns20t8	-2.29333	5.80551	1.000	-49.3918	44.8052
	ns20t12	14.73667	6.68893	.794	-30.9884	60.46.7
	ns40t0	-21.17333	5.12882	.361	-84.3193	41.9726
	ns40t2	-8.10333	8.73598	1.000	-70.2347	54.0280
	ns40t8	1.29667	6.35745	1.000	-43.7683	46.3617
	ns40t12	7.67667	5.18265	.959	53.0359	68.3892
	ns60t0	-21.17333	5.12882	.361	-84.3193	41.9726
	ns60t2	-11.61667	6.81555	.346	-57.8329	34.5996
	ns60t4	-7.08000	6.51550	.399	-52.3337	38.1737
	ns60t8	-2.13000	6.40029	1.000	-47.2208	42.9608
	ns60t12	11.74000	6.30416	.903	-33.3216	56.8016

Table F30 Multiple comparion of the percentage of gallic acid under storage condition at room temperature (30ºC) by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable: content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
ns40t8	ns20 t0	-22.47000	3.75665	.194	-68.7218	23.7818
	ns20 t2	-11.11333	4.82570	.757	-44.3824	22.1557
	ns20t4	-10.25000	9.10037	.997	-89.6255	59.1255
	ns20t8	-3.59000	4.63806	1.000	-36.5941	29.4141
	ns20t12	13.44000	5.70521	.737	-25.2489	52.1289
	ns40t0	-22.47000	3.75665	.194	-68.7218	23.7818
	ns40t2	-9.40000	8.00781	.997	-74.3176	55.5176
	ns40t4	-1.29667	6.35745	1.000	-46.3617	43.7683
	ns40t12	6.38000	3.82982	.925	-36.7408	49.5008
	ns60t0	-22.47000	3.75665	.194	68.7218	23.7818
	ns60t2	-12.91333	5.85315	.793	-52.9276	27.1010
	ns60t4	-8.37667	5.50086	.977	-45.3848	28.6315
	ns60t8	-3.42667	5.36390	1.000	-39.4227	32.5694
	ns60t12	10.44333	5.24882	.870	-24.7855	45.6722
ns40t12	ns20 t0	-28.85000*	.74505	.005	-38.0231	-19.6769
	ns20 t2	-17.49333	3.11931	.194	-51.1528	16.1661
	ns20t4	16.63000	8.32222	.837	-117.1389	83.8489
	ns20t8	9.97000	2.82034	.434	-39.5384	19.6484
	ns20t12	7.06000	4.35800	.934	-42.9837	57.1037
	ns40t0	-28.85000*	.74505	.005	-38.0231	-19.6769
	ns40t2	-15.78000	7.11110	.774	-101.0252	69.4652
	ns40t4	-7.67667	5.18265	.959	-68.3892	53.0359
	ns40t8	-6.38000	3.82982	.925	-49.5008	36.7408
	ns60t0	-28.85000*	.74505	.005	-38.0231	-19.6769
	ns60t2	-19.29333	4.54996	.336	-71.8341	33.2475
	ns60t4	-14.75667	4.08682	.431	-61.2564	31.7430
	ns60t8	-9.80667	3.90053	.688	-53.8592	34.2459
	ns60t12	4.06333	3.74069	.996	-37.8808	46.0075
ns60t0	ns20 t0	.00000	.00000	.	.0000	.0000
	ns20 t2	11.35667	3.02903	.418	25.9367	48.6501
	ns20t4	12.22000	8.28881	.958	-89.8318	114.2718
	ns20t8	13.88000	2.72015	.148	-14.6105	52.3705
	ns20t12	35.91000	4.29384	.105	-16.9557	88.7757
	ns40t0	.00000	.00000		.0000	.0000
	ns40t2	13.07000	7.07196	.877	-74.0000	100.1400
	ns40t4	21.17333	5.12882	.361	-41.9726	84.3193
	ns40t8	22.47000	3.75665	.194	-23.7818	68.7218
	ns40t12	28.85000*	.74505	.005	19.6769	38.0231
	ns60t2	9.55667	4.48854	.800	-45.7063	64.8196
	ns60t4	14.09333	4.01833	.460	-35.3804	63.5671
	ns60t8	19.04333	3.82871	.267	-28.0957	66.1824
	ns60t12	32.91333	3.66574	.092	-12.2193	78.0460

Table F30 Multiple comparion of the percentage of gallic acid under storage
condition at room temperature (30ºC) by Dunett 3T test (continued)

Multiple Comparisons

Dependent Variable content
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std Error	Sig	95% Confidence Interval	
					Lower Bound	Upper Bound
ns60t2	ns20 t0	-9.55667	4.48854	.800	-64.8196	45.7063
	ns20 t2	1.80000	5.41498	1.000	-37.7368	41.3368
	ns20t4	2.66333	9.42610	1.000	-73.0739	78.4006
	ns20t8	9.32333	5.24845	.921	-30.7508	49.3974
	ns20t12	26.35333	6.21161	.216	-15.3719	68.0786
	ns40t0	-9.55667	4.48854	.800	-64.8196	45.7063
	ns40t2	3.51333	8.37614	1.000	-59.1828	66.2094
	ns40t4	11.61667	6.81555	.946	-34.5996	57.8329
	ns40t8	12.91333	5.85315	.793	-27.1010	52.9276
	ns40t12	19.29333	4.54996	.336	-33.2475	71.8341
	ns60t0	-9.55667	4.48854	.800	-64.8196	45.7063
	ns60t4	4.53667	6.02445	1.000	-36.1814	45.2547
	ns60t8	9.48667	5.89966	.964	-30.6956	49.6639
	ns60t12	23.35667	5.79523	.256	-16.4749	63.1832
ns60t4	ns20 t0	-14.09333	4.01833	.460	-63.5671	35.3804
	ns20 t2	-2.73667	5.03210	1.000	-38.0812	32.6079
	ns20t4	-1.87333	9.21148	1.000	-79.7847	76.0380
	ns20t8	4.78667	4.85245	1.000	-30.6003	40.1736
	ns20t12	21.81667	5.88082	.310	-17.7445	61.3778
	ns40t0	-14.09333	4.01833	.460	-63.5671	35.3804
	ns40t2	1.02333	8.13386	1.000	-64.9481	62.9014
	ns40t4	7.08000	6.51550	.999	-38.1737	52.3337
	ns40t8	8.37667	5.50086	.977	-28.6315	45.3848
	ns40t12	14.75667	4.08682	.431	-31.7430	61.2564
	ns60t0	-14.09333	4.01833	.460	-63.5671	35.3804
	ns60t2	-4.53667	6.02445	1.000	-45.2547	36.1814
	ns60t8	4.95000	5.55032	1.000	-32.3415	42.2415
	ns60t12	18.82000	5.43918	.368	-17.8588	55.4988
ns60t8	ns20 t0	-19.04333	3.82871	.267	-66.1824	28.0957
	ns20 t2	-7.68667	4.88201	.968	-41.5067	26.1334
	ns20t4	-6.82333	9.13035	1.000	-85.7782	72.1316
	ns20t8	-.16333	4.69662	1.000	-33.8054	33.4787
	ns20t12	16.86667	5.75292	.524	-22.0369	55.7702
	ns40t0	-19.04333	3.82871	.267	-66.1824	28.0957
	ns40t2	-5.97333	8.04187	1.000	-70.5954	58.6488
	ns40t4	2.13000	6.40029	1.000	-42.9608	47.2208
	ns40t8	3.42667	5.36390	1.000	-32.5694	39.4227
	ns40t12	9.80667	3.90053	.688	-34.2459	53.8592
	ns60t0	-19.04333	3.82871	.267	-66.1824	28.0957
	ns60t2	-9.48667	5.89966	.964	-49.6689	30.6956
	ns60t4	-4.95000	5.55032	1.000	-42.2415	32.3415
	ns60t12	13.87000	5.30063	.637	-21.7344	49.4744

APPENDIX G

Permeation study

Table G1 The percentage of cumulative permeation of gallic acid from emblica

extract solution

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.051	0.026	0.029	0.035	0.013
1	0.052	0.034	0.032	0.040	0.011
2	0.056	0.040	0.037	0.044	0.010
4	0.062	0.044	0.041	0.049	0.011
6	0.063	0.047	0.044	0.051	0.010
8	0.067	0.050	0.047	0.054	0.011
10	0.068	0.052	0.049	0.057	0.010
12	0.070	0.056	0.053	0.059	0.009
16	0.079	0.061	0.057	0.066	0.012
20	0.084	0.065	0.061	0.070	0.012
24	0.085	0.067	0.063	0.072	0.012

Table G2 The percentage of cumulative permeation of N2064 formulation

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.279	0.223	0.099	0.214	0.092
1	0.284	0.234	0.103	0.256	0.094
2	0.313	0.254	0.117	0.311	0.100
4	0.342	0.272	0.130	0.363	0.108
6	0.351	0.279	0.137	0.404	0.109
8	0.359	0.289	0.136	0.441	0.114
10	0.371	0.297	0.145	0.479	0.115
12	0.397	0.313	0.154	0.522	0.123
16	0.475	0.356	0.196	0.589	0.140
20	0.563	0.420	0.270	0.680	0.146
24	0.658	0.485	0.353	0.774	0.153

Table G3 The percentage of cumulative permeation of N4064 formulation

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.245	0.220	0.239	0.235	0.013
1	0.248	0.226	0.232	0.236	0.012
2	0.233	0.225	0.225	0.228	0.004
4	0.254	0.229	0.235	0.239	0.013
6	0.263	0.235	0.248	0.249	0.014
8	0.275	0.240	0.261	0.259	0.018
10	0.284	0.247	0.288	0.273	0.023
12	0.303	0.259	0.306	0.289	0.026
16	0.338	0.275	0.385	0.333	0.055
20	0.357	0.299	0.501	0.386	0.104
24	0.397	0.338	0.503	0.413	0.083

Table G4 The percentage of cumulative permeation of N6064 formulation

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.042	0.094	0.023	0.053	0.037
1	0.045	0.099	0.030	0.058	0.036
2	0.048	0.105	0.036	0.063	0.037
4	0.049	0.109	0.050	0.069	0.034
6	0.050	0.104	0.051	0.068	0.031
8	0.053	0.107	0.058	0.073	0.030
10	0.055	0.104	0.073	0.077	0.025
12	0.070	0.105	0.092	0.089	0.018
16	0.074	0.121	0.131	0.109	0.030
20	0.111	0.166	0.203	0.160	0.046
24	0.152	0.227	0.303	0.227	0.075

Table G5 The percentage of cumulative permeation per area of emblica extract serum

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.027	0.017	0.014	0.020	0.007
1	0.029	0.021	0.020	0.023	0.005
2	0.031	0.023	0.022	0.026	0.005
4	0.035	0.027	0.025	0.029	0.005
6	0.037	0.028	0.026	0.030	0.006
8	0.039	0.030	0.028	0.032	0.006
10	0.040	0.031	0.029	0.033	0.006
12	0.040	0.033	0.031	0.035	0.005
16	0.044	0.039	0.033	0.039	0.006
20	0.046	0.044	0.035	0.041	0.006
24	0.047	0.048	0.036	0.044	0.007

Table G6 The percentage of cumulative permeation per area of NS2064 formulation

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.128	0.141	0.232	0.167	0.057
1	0.127	0.165	0.393	0.229	0.144
2	0.133	0.174	0.390	0.232	0.138
4	0.140	0.181	0.395	0.238	0.137
6	0.144	0.182	0.396	0.240	0.136
8	0.150	0.188	0.398	0.245	0.134
10	0.154	0.191	0.407	0.251	0.137
12	0.157	0.192	0.407	0.252	0.136
16	0.164	0.201	0.408	0.258	0.131
20	0.165	0.228	0.411	0.268	0.128
24	0.176	0.313	0.415	0.301	0.120

Table G7 The percentage of cumulative permeation per area of NS4064 formulation

Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.158	0.138	0.102	0.133	0.028
1	0.170	0.146	0.107	0.141	0.032
2	0.187	0.165	0.114	0.156	0.038
4	0.189	0.164	0.115	0.156	0.038
6	0.195	0.166	0.120	0.160	0.038
8	0.182	0.158	0.106	0.149	0.039
10	0.189	0.158	0.107	0.151	0.042
12	0.183	0.153	0.108	0.148	0.038
16	0.191	0.156	0.110	0.152	0.041
20	0.203	0.158	0.112	0.158	0.045
24	0.225	0.160	0.125	0.170	0.050

Table G8 The percentage of cumulative permeation per area of NS6064 formulation

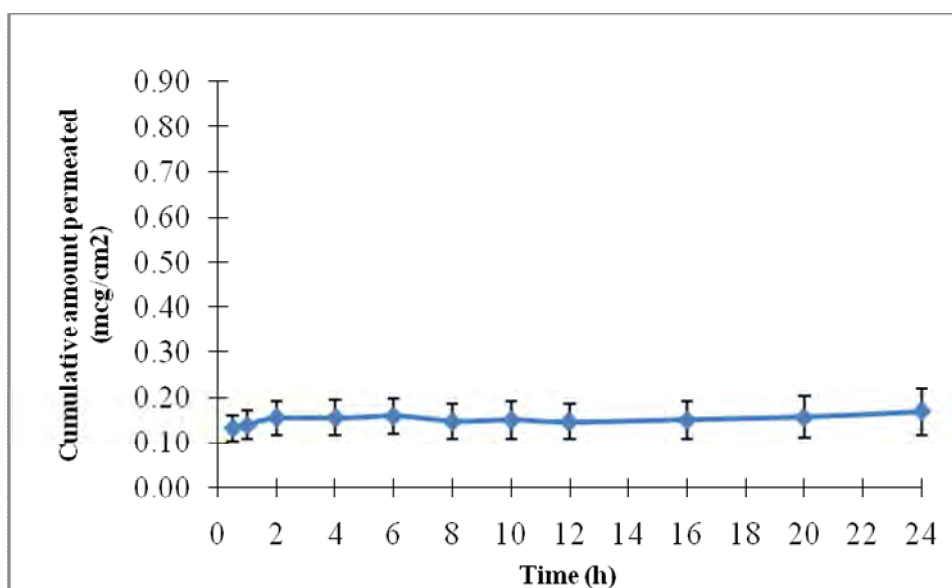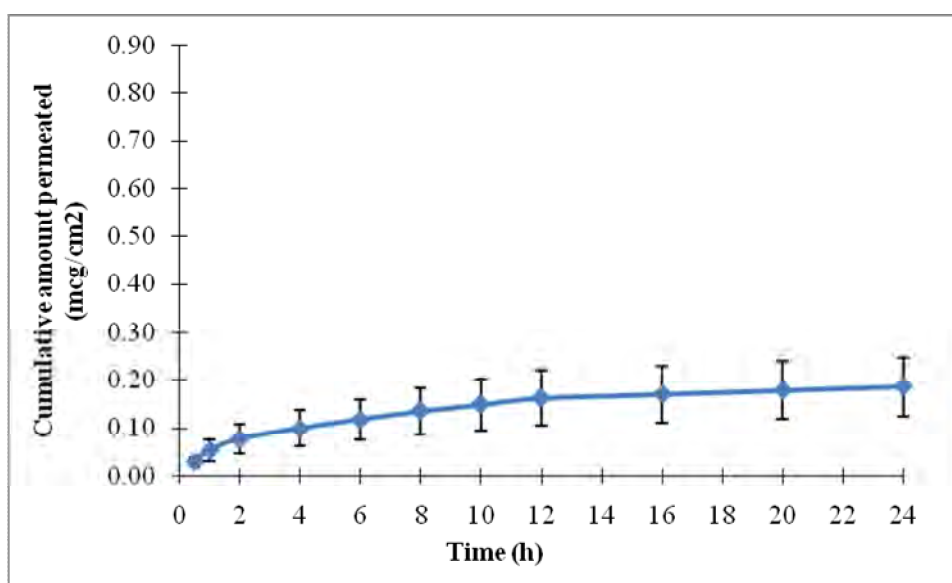| Time	Cumulative permeation of gallic acid per area				
	N1	N2	N3	Mean	SD
0.5	0.023	0.024	0.041	0.029	0.010
1	0.037	0.043	0.079	0.053	0.022
2	0.055	0.064	0.113	0.077	0.031
4	0.070	0.086	0.143	0.100	0.038
6	0.086	0.102	0.165	0.118	0.042
8	0.098	0.117	0.190	0.135	0.048
10	0.109	0.129	0.210	0.149	0.053
12	0.118	0.139	0.227	0.161	0.058
16	0.125	0.147	0.237	0.170	0.059
20	0.133	0.155	0.247	0.178	0.061
24	0.141	0.161	0.256	0.186	0.062
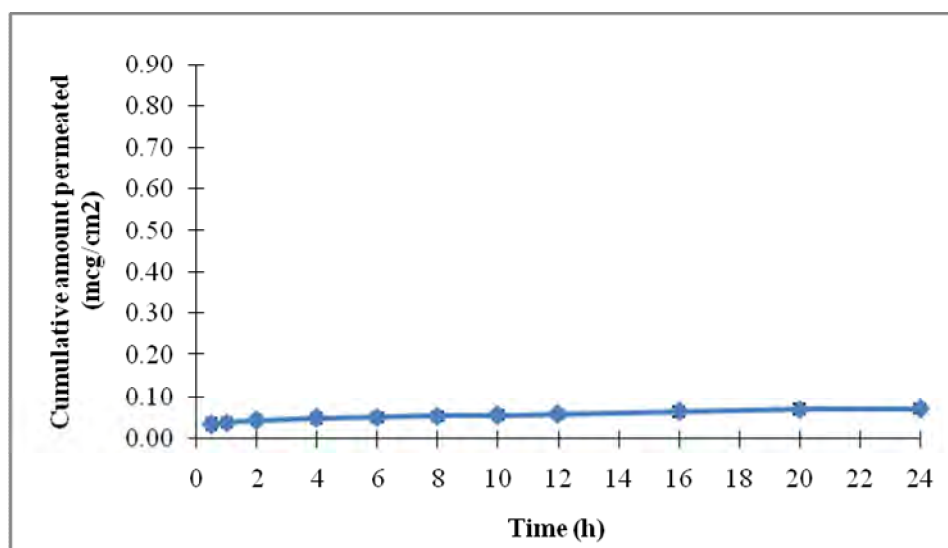
(a)
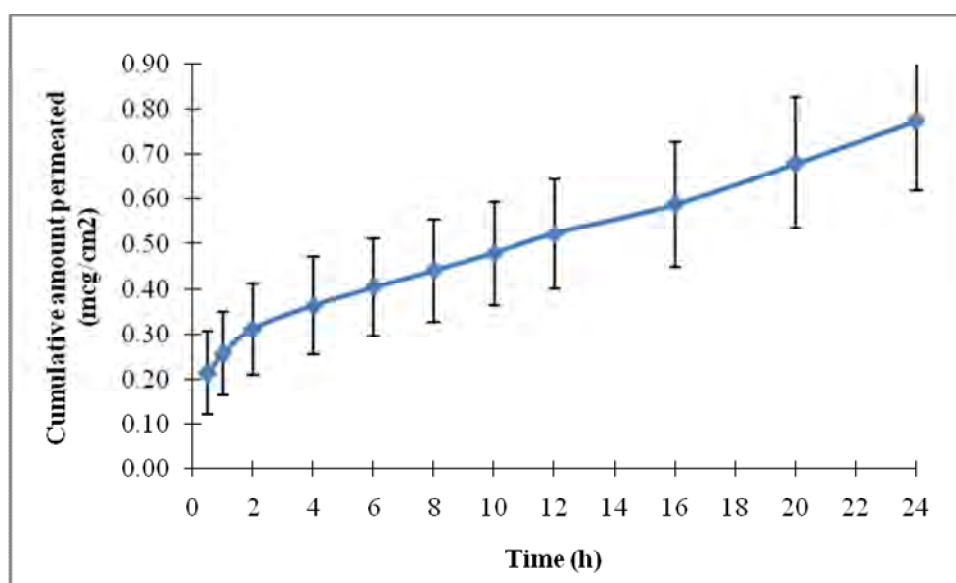
(b)

Figure G1 The permeation profile of gallic acid from (a) emblica extract solution, (b) N2064, (c) N4064, (d) N6064, (e) emblica extract serum, (f) NS2064, (g) NS4064, (h) NS6064, (mean±SD, n=3)

188
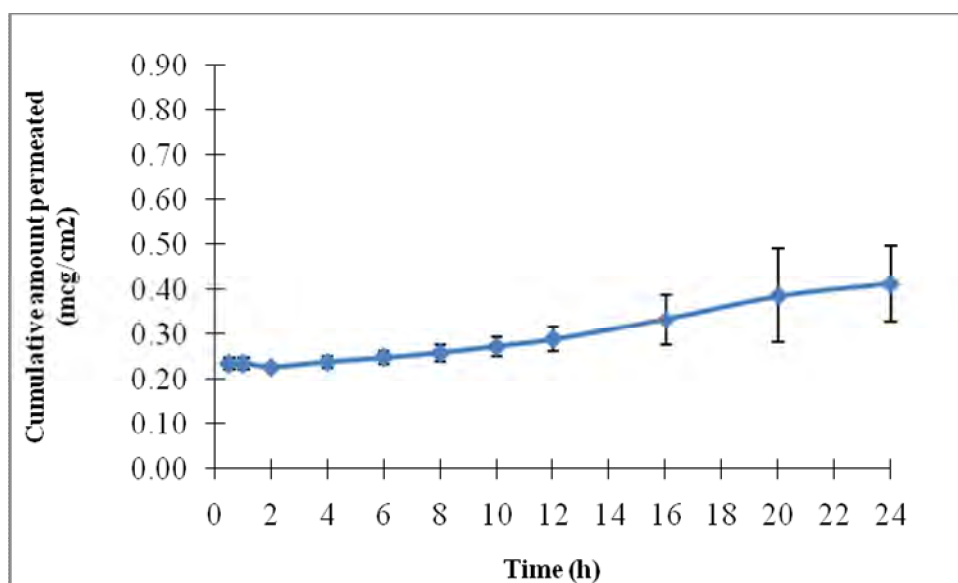
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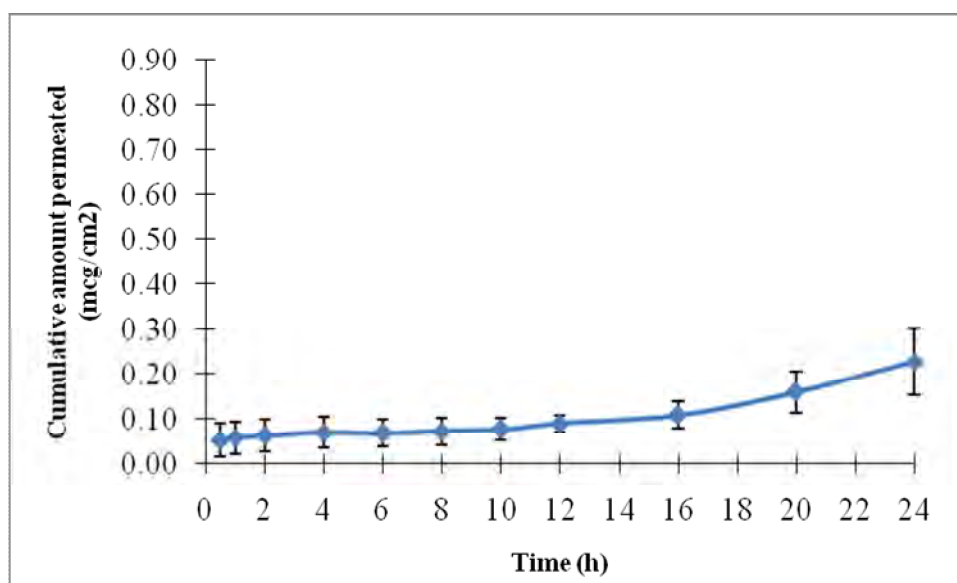
(d)

Figure G1 The permeation profile of gallic acid from (a) emblica extract solution, (b)
N2064, (c) N4064, (d) N6064, (e) emblica extract serum, (f) NS2064, (g) NS4064, (h)
NS6064, (mean±SD, n=3)
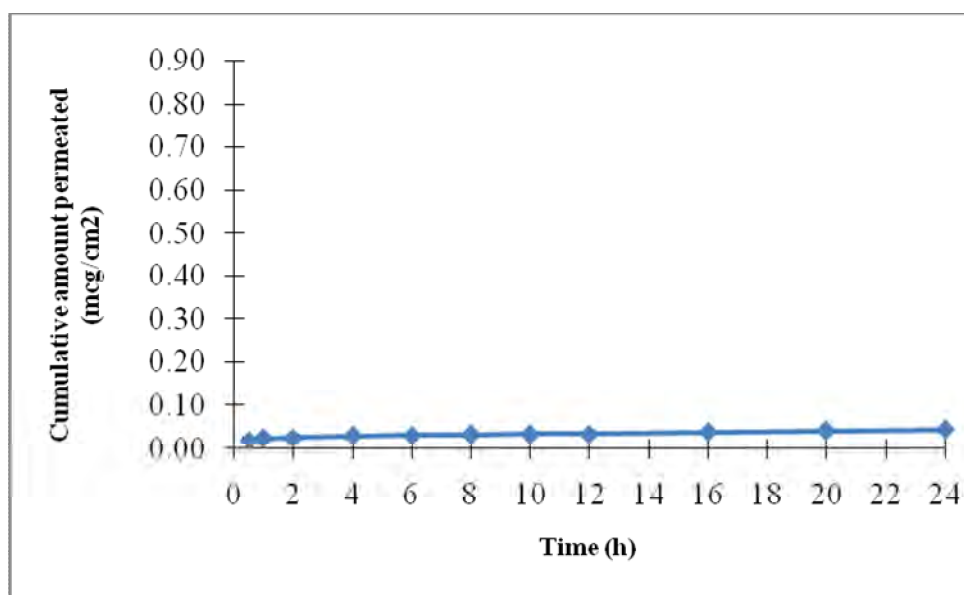
(e)
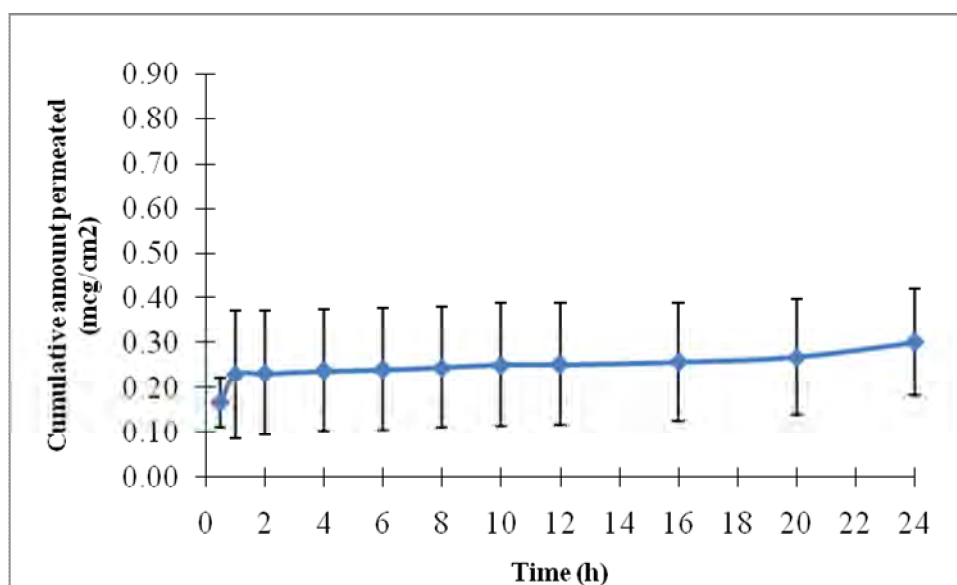
(f)

Figure G1 The permeation profile of gallic acid from (a) emblica extract solution, (b) N2064, (c) N4064, (d) N6064, (e) emblica extract serum, (f) NS2064, (g) NS4064, (h) NS6064, (mean±SD, n=3)

(g)

(h)

Figure G1 The permeation profile of gallic acid from (a) emblica extract solution, (b) N2064, (c) N4064, (d) N6064, (e) emblica extract serum, (f) NS2064, (g) NS4064, (h) NS6064, (mean±SD, n=3)
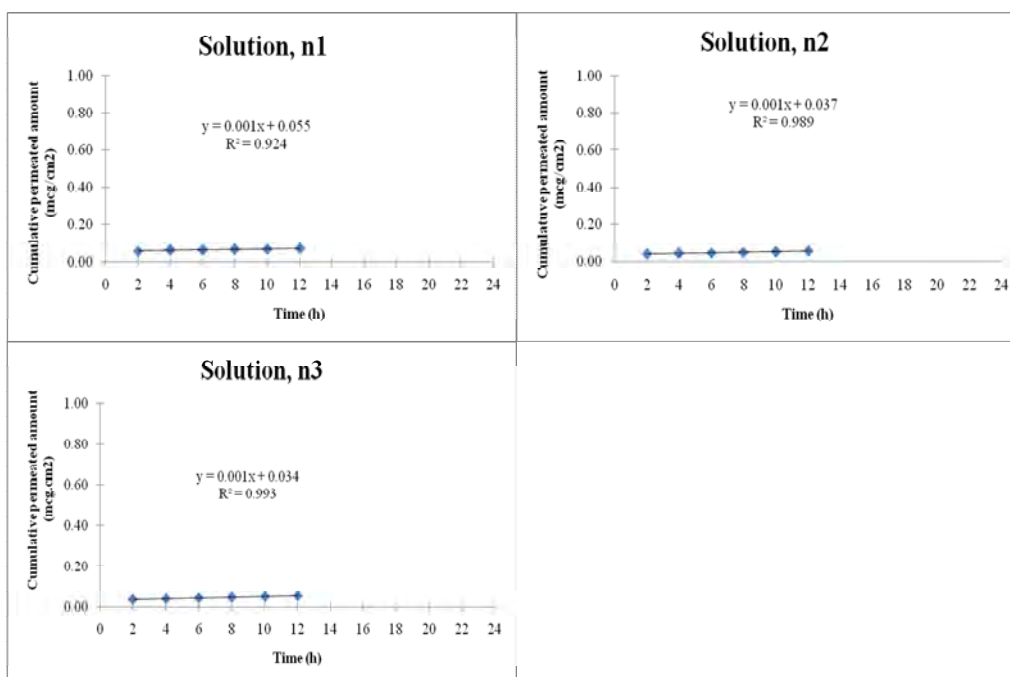
Figure G2 Cumulative permeation of gallic acid per unit area as a function of time from emblica extract solution formulation (n=3)
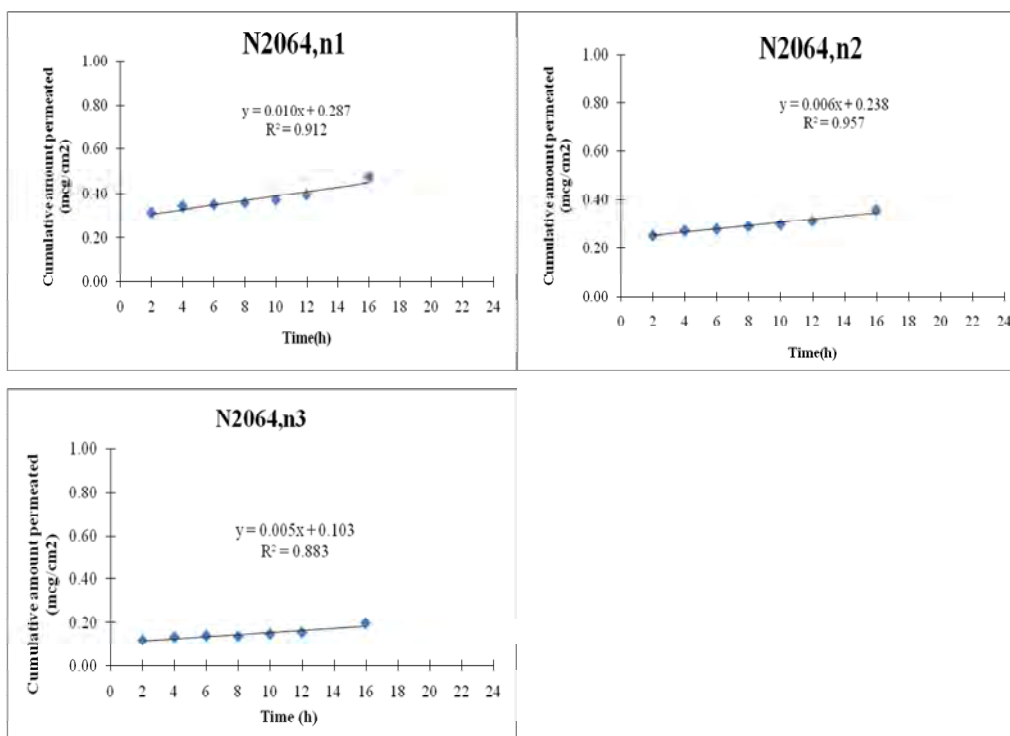

Figure G3 Cumulative permeation of gallic acid per unit area as a function of time from N2064 formulation (n=3)
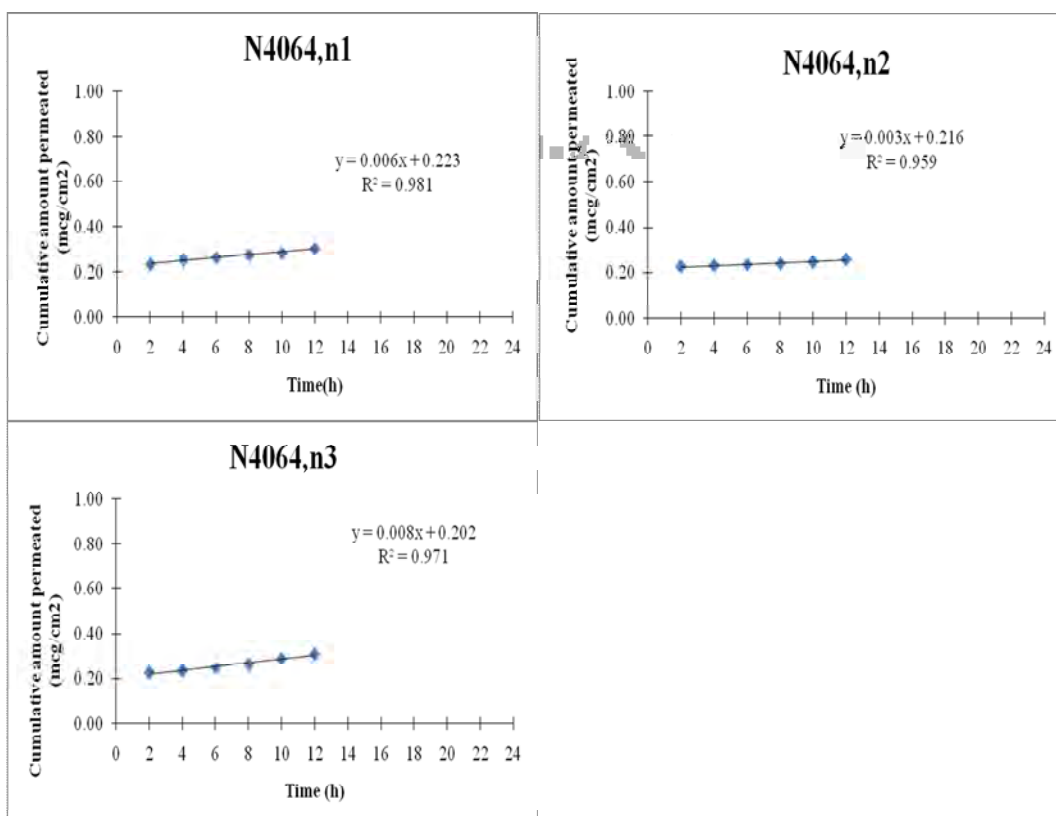
Figure G4 Cumulative permeation of gallic acid per unit area as a function of time from N4064 formulation (n=3)

Figure G5 Cumulative permeation of gallic acid per unit area as a function of time from N6064 formulation (n=3)

Figure G6 Cumulative permeation of gallic acid per unit area as a function of time from emblica serum formulation (n=3)

Figure G7 Cumulative permeation of gallic acid per unit area as a function of time from NS2064 formulation (n=3)

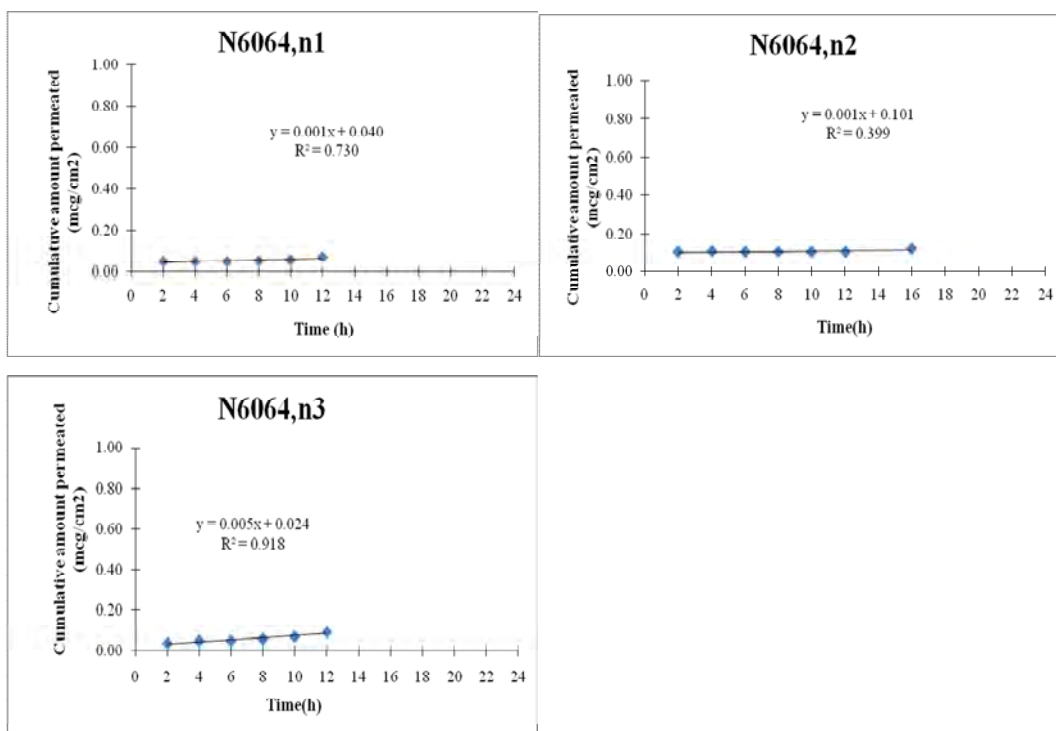Figure G8 Cumulative permeation of gallic acid per unit area as a function of time from NS4064 formulation (n=3)

Figure G9 Cumulative permeation of gallic acid per unit area as a function of time from NS6064 formulation (n=3)
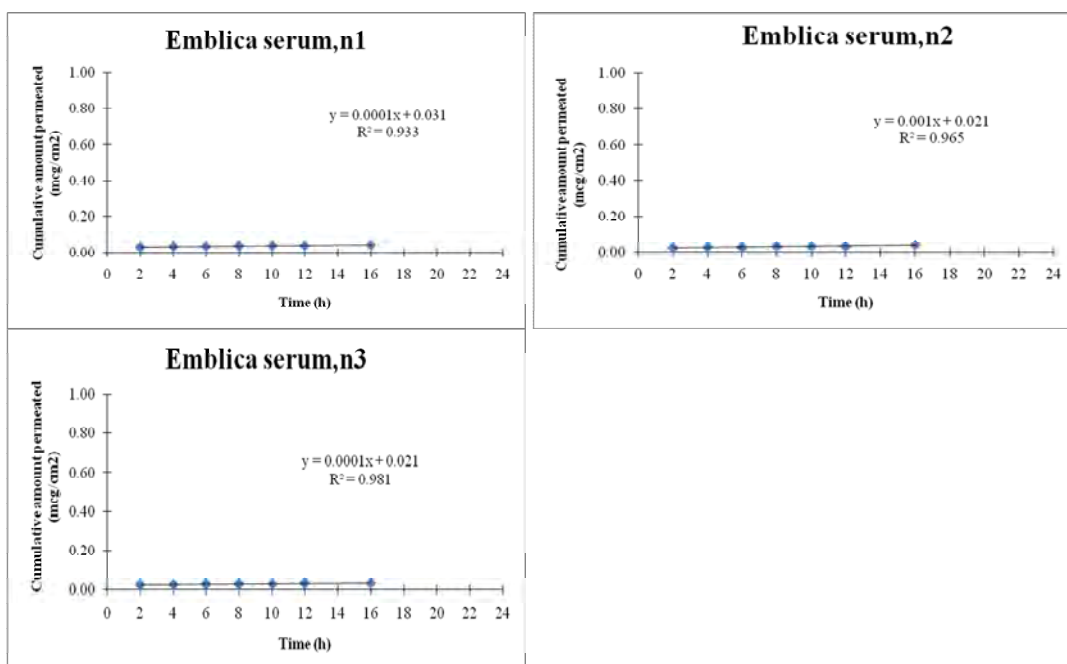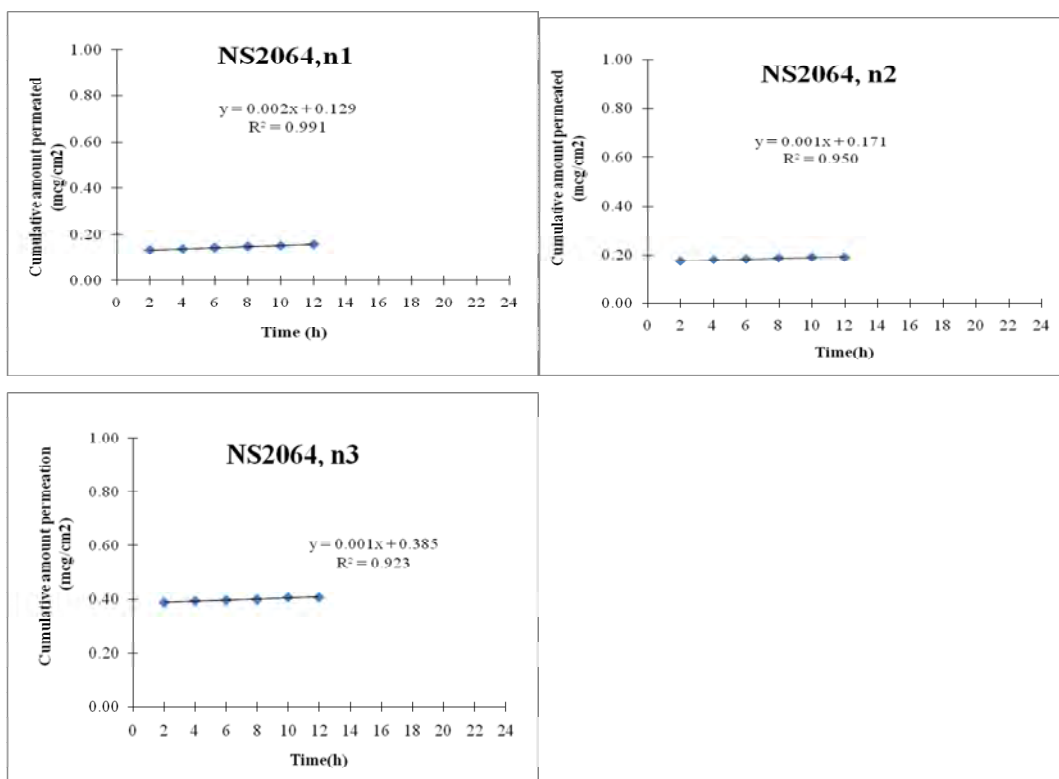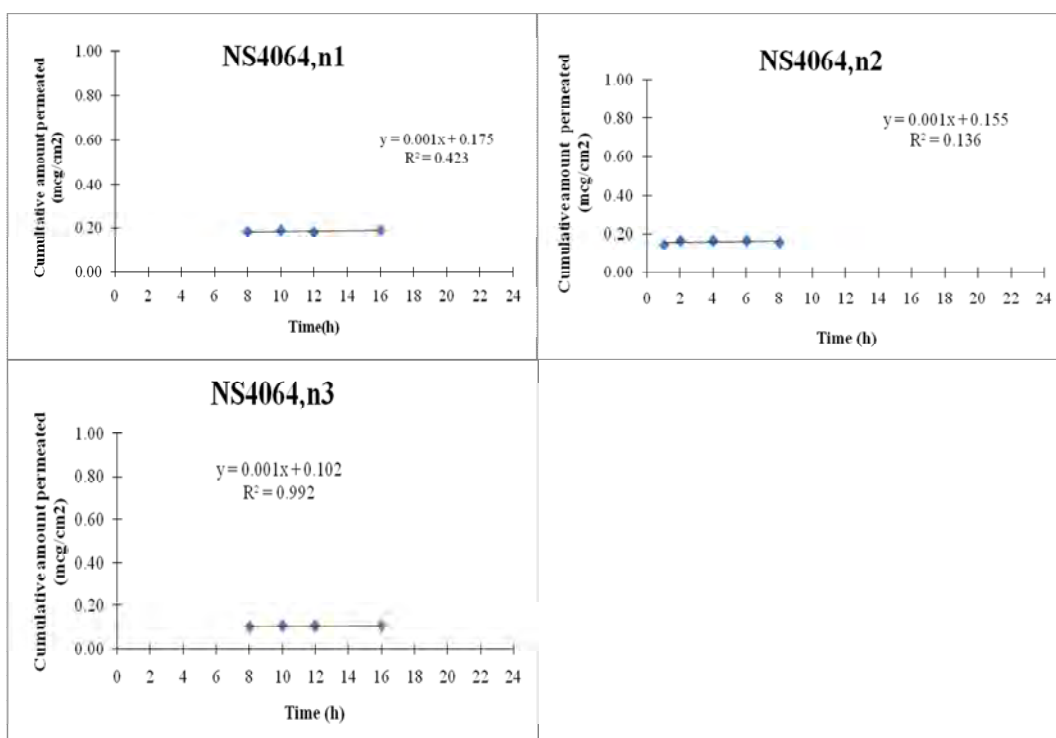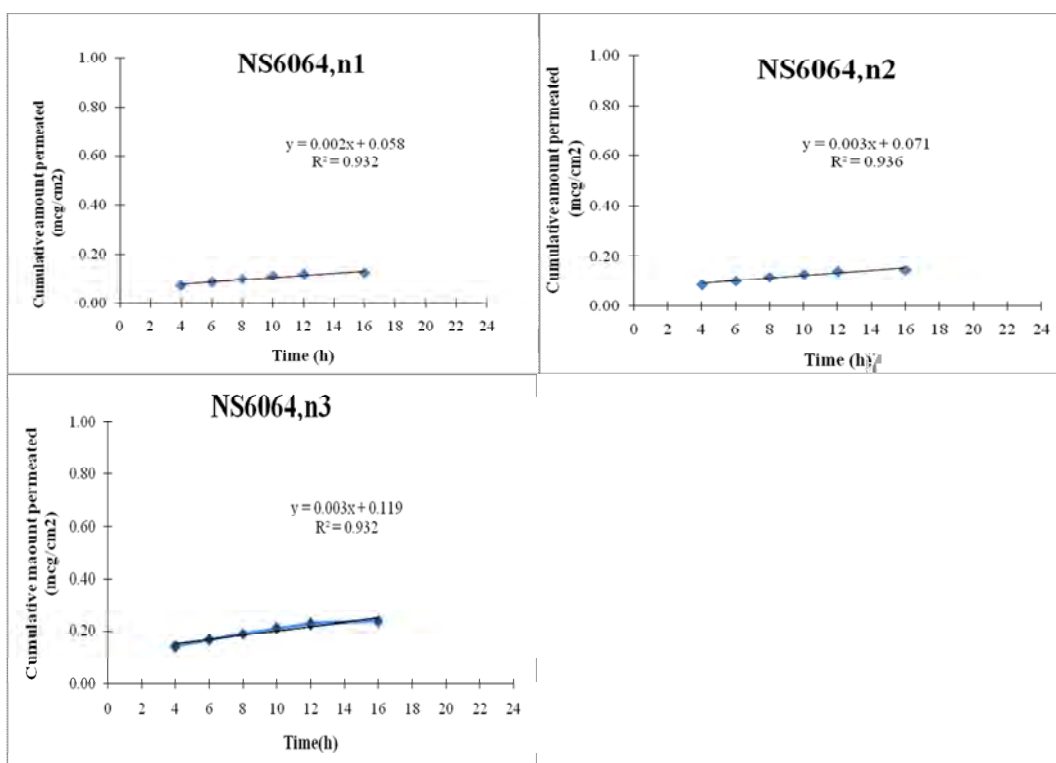
Table G9 The membrane permeability coefficients of various formulation of gallic acid

Formulation	slope	Cd(mg/ml)	Papp	% drug deposited into the skin
Solution				
n1	0.0010	3.12	0.0003	2.37
n2	0.0010	3.12	0.0003	2.26
n3	0.0010	3.12	0.0003	1.25
mean	0.0010		0.0003	1.96
SD	0.0000		0.0000	0.62
N2064				
n1	0.0100	2.82	0.0035	3.14
n2	0.0060	2.82	0.0021	2.32
n3	0.0050	2.82	0.0018	3.46
mean	0.0070		0.0025	2.97
SD	0.0026		0.0009	0.59
N4064				
n1	0.0060	2.82	0.0021	0.92
n2	0.0030	2.82	0.0011	1.49
n3	0.0080	2.82	0.0028	2.54
	0.0057		0.0020	1.65
SD	0.0025		0.0009	0.83
N6064				
n1	0.0010	2.82	0.0004	3.50
n2	0.0010	2.82	0.0004	2.14
n3	0.0050	2.82	0.0018	2.00
mean	0.0023		0.0008	2.55
SD	0.0023		0.0008	0.83

Table G9 The membrane permeability coefficients of various formulation of gallic acid (continued)

Formulation	slope	Cd(mg/ml)	Papp	% drug deposited into the skin
serum				
n1	0.0001	1.32	0.0001	2.72
n2	0.0001	1.32	0.0001	2.17
n3	0.0001	1.32	0.0001	1.22
mean	0.0001		0.0001	2.04
SD	0.0000		0.0000	0.76
NS2064				
n1	0.0020	1.32	0.0015	0.08
n2	0.0010	1.32	0.0008	0.12
n3	0.0010	1.32	0.0008	0.08
mean	0.0013		0.0010	0.09
SD	0.0006		0.0004	0.02
NS4064				
n1	0.0010	1.31	0.0008	0.76
n2	0.0010	1.32	0.0008	0.31
n3	0.0010	1.31	0.0008	0.10
mean	0.0010		0.0008	0.39
SD	0.0000		0.0000	0.34
NS6064				
n1	0.0020	1.33	0.0015	0.10
n2	0.0030	1.35	0.0022	0.17
n3	0.0030	1.33	0.0023	0.13
mean	0.0027		0.0020	0.13
SD	0.0006		0.0004	0.03

Table G10 Test of homogeneity of variacnes

Test of Homogeneity of Variances

papp

Levene Statistic	df1	df2	Sig.
3.702	7	16	.014

Table G11 Multiple comparion of the permeability coefficient values of formulations by Dunett T3 test

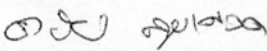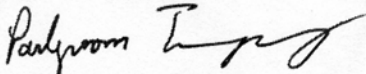**Multiple Comparisons**

Dependent Variable: papp
Dunnett T3

(I) code	(J) code	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
solution	n2064	-.0021667	.0005239	.268	-.007571	.003238
	n4064	-.0017000	.0004933	.359	-.006789	.003389
	n6064	-.0005667	.0004667	.949	-.005381	.004247
	emblica serum	.0002000	.0000000	.	.000200	.000200
	ns2064	-.0007333	.0002333	.412	-.003140	.001674
	ns4064	-.0005000	.0000000	.	-.000500	-.000500
	ns6064	-.0012000	.0004619	.534	-.005965	.003565
n2064	solution	.0021667	.0005239	.268	-.003238	.007571
	n4064	.0004667	.0007196	1.000	-.003634	.004567
	n6064	.0016000	.0007016	.581	-.002420	.005620
	emblica serum	.0023667	.0005239	.231	-.003038	.007771
	ns2064	.0014333	.0005735	.528	-.002812	.005678
	ns4064	.0016667	.0005239	.405	-.003738	.007071
	ns6064	.0009667	.0006984	.937	-.003041	.004975
n4064	solution	.0017000	.0004933	.359	-.003389	.006789
	n2064	-.0004667	.0007196	1.000	-.004567	.003634
	n6064	.0011333	.0006791	.843	-.002735	.005001
	emblica serum	.0019000	.0004933	.301	-.003189	.006989
	ns2064	.0009667	.0005457	.793	-.002966	.004899
	ns4064	.0012000	.0004933	.579	-.003889	.006289
	ns6064	.0005000	.0006758	1.000	-.003352	.004352
n6064	solution	.0005667	.0004667	.949	-.004247	.005381
	n2064	-.0016000	.0007016	.581	-.005620	.002420
	n4064	-.0011333	.0006791	.843	-.005001	.002735
	emblica serum	.0007667	.0004667	.830	-.004047	.005581
	ns2064	-.0001667	.0005217	1.000	-.003834	.003500
	ns4064	.0000667	.0004667	1.000	-.004747	.004881
	ns6064	-.0006333	.0006566	.996	-.004367	.003100
emblica serum	solution	-.0002000	.0000000	.	-.000200	-.000200
	n2064	-.0023667	.0005239	.231	-.007771	.003038
	n4064	-.0019000	.0004933	.301	-.006989	.003189
	n6064	-.0007667	.0004667	.830	-.005581	.004047
	ns2064	-.0009333	.0002333	.284	-.003340	.001474
	ns4064	-.0007000	.0000000	.	-.000700	-.000700
	ns6064	-.0014000	.0004619	.434	-.006165	.003365
ns2064	solution	.0007333	.0002333	.412	-.001674	.003140
	n2064	-.0014333	.0005735	.528	-.005678	.002812
	n4064	-.0009667	.0005457	.793	-.004899	.002966
	n6064	.0001667	.0005217	1.000	-.003500	.003834
	emblica serum	.0009333	.0002333	.284	-.001474	.003340
	ns4064	.0002333	.0002333	.983	-.002174	.002640
	ns6064	-.0004667	.0005175	.996	-.004087	.003153
ns4064	solution	.0005000	.0000000	.	.000500	.000500
	n2064	-.0016667	.0005239	.405	-.007071	.003738
	n4064	-.0012000	.0004933	.579	-.006289	.003889
	n6064	-.0000667	.0004667	1.000	-.004881	.004747
	emblica serum	.0007000	.0000000	.	.000700	.000700
	ns2064	-.0002333	.0002333	.983	-.002640	.002174
	ns6064	-.0007000	.0004619	.870	-.005465	.004065
ns6064	solution	.0012000	.0004619	.534	-.003565	.005965
	n2064	-.0009667	.0006984	.937	-.004975	.003041
	n4064	-.0005000	.0006758	1.000	-.004352	.003352
	n6064	.0006333	.0006566	.996	-.003100	.004367
	emblica serum	.0014000	.0004619	.434	-.003365	.006165
	ns2064	.0004667	.0005175	.996	-.003153	.004087
	ns4064	.0007000	.0004619	.870	-.004065	.005465

APPENDIX H

Study Protocol Approval by Chulalongkorn University
Animal Care and Use Committee, Bangkok, Thailand

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	□ Original	□ Renew
Animal Use Protocol No. 10-33-011	**Approval No. 10-33-011**	

Protocol Title
Formulation of niosomes containing *Phyllanthus emblica* extract with whitening and free radical scavenging activities

Principal Investigator
Suchada Chutimaworapan, Ph.D.

Certification of Institutional Animal Care and Use Committee (IACUC)
This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval May 17, 2010	**Date of Expiration** May 17, 2011

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan
BKK-THAILAND. 10330

Signature of Chairperson	**Signature of Authorized Official**
Name and Title THONGCHAI SOOKSAWATE, Ph.D. Chairman	**Name and Title** PARKPOOM TENGAMNUAY, Ph.D. Associate Dean (Research and Academic Service)

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

VITA

Miss Saowalak Leartamonstiean was born on April 30, 1981 in Chainart, Thailand. She received her Bachelor's degree in Pharmacy from the Faculty of Pharmaceutical Sciences, Mahidol University, Bangkok, Thailand in 2003. Since graduation, she has worked at the Food and Drug Administration, Ministry of Public Health, Nonthaburi. She enrolled the Master's degree program in Pharmacy at Chulalongkorn University in 2008.