IN VITRO COMPARATIVE EVALUATION OF VARIOUS NANOSYSTEMS CONTAINING *PHYLLANTHUS EMBLICA* FRUIT EXTRACT

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmaceutical Technology

Department of Pharmaceutics and Industrial Pharmacy

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2012

การประเมินเปรียบเทียบแบบนอกกายของระบบนาโนชนิดต่าง ๆ ที่บรรจุสารสกัดผลมะขามป้อม

นางสาวเศฐินี จันทร์ภิรมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

Thesis Title	*IN VITRO* COMPARATIVE EVALUATION OF VARIOUS NANOSYSTEMS CONTAINING *PHYLLANTHUS EMBLICA* FRUIT EXTRACT
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เศฐินี จันทร์ภิรมย์: การประเมินเปรียบเทียบแบบนอกกายของระบบนาโนชนิดต่างๆที่บรรจุสารสกัดผลมะขามป้อม. (*IN VITRO* COMPARATIVE EVALUATION OF VARIOUS NANOSYSTEMS CONTAINING *PHYLLANTHUS EMBLICA* FRUIT EXTRACT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร.ดุษฎี ชาญวาณิช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.วราภรณ์ สุวกูล, อ. ดร.อัญญพร ตันศิริคงคล, 147 หน้า.

สารสกัดจากผลมะขามป้อมเป็นสารที่มีฤทธิ์ต้านอนุมูลอิสระและทำให้ผิวกระจ่างใสที่รู้จักกันอย่างแพร่หลาย การสกัดด้วยวิธีที่แตกต่างกันจะให้สารสกัดที่มีฤทธิ์ทางชีวภาพต่างกัน ดังนั้น งานวิจัยนี้จึงมีจุดประสงค์เพื่อหาสารสกัดจากผลมะขามป้อมที่มีฤทธิ์ดีที่สุดและเสนอระบบที่เหมาะสมที่สุดสำหรับนำส่งทางผิวหนัง สารสกัดที่มีฤทธิ์ดีที่สุดจะถูกเลือกจากการศึกษาฤทธิ์ทางชีวภาพและปริมาณฟีนอลลิกทั้งหมดของสารสกัดจากผลมะขามป้อมที่เตรียมด้วยวิธีการสกัดที่ต่างกัน สารสกัดออกฤทธิ์ที่เลือกจะถูกนำมาบรรจุในระบบนาโนชนิดต่าง ๆ ได้แก่ ลิโพโซมชนิดธรรมดา อิลาสติกลิโพโซม นิโอโซม ไมโครอิมัลชัน (ME1, ME2, ME3) และนาโนอิมัลชัน (NE1, NE2, NE3) ระบบนาโนดังกล่าวจะถูกประเมินทางด้านลักษณะภายนอก ขนาดอนุภาค และประสิทธิภาพการกักเก็บ ศึกษาความคงตัวของระบบ การปลดปล่อยแบบนอกกายและการซึมผ่านผิวหนังแบบนอกกาย ผลการศึกษา พบว่า สารสกัดเอทานอลจากผลมะขามป้อมแห้งมีฤทธิ์ต้านอนุมูลอิสระ (IC_{50} = 1.221 ± 0.005 ไมโครกรัมต่อมิลลิลิตร) ฤทธิ์ต้านเอนไซม์ไทโรซิเนส (IC_{50} = 0.519 ± 0.007 มิลลิกรัมต่อมิลลิลิตร) ปริมาณฟีนอลลิกทั้งหมด (490.756 ± 0.185 มิลลิกรัมสมมูลย์กับกรดแกลลิคต่อกรัมของสารสกัด) สูง และมีปริมาณที่เตรียมได้สูง ระบบนาโนชนิดต่าง ๆ ที่บรรจุสารสกัดมีความคงตัวดีทั้งทางกายภาพและทางชีวภาพเมื่อเก็บไว้ที่อุณหภูมิ 4 องศาเซลเซียส (สำหรับลิโพโซม) หรือ ที่อุณหภูมิห้อง (สำหรับนิโอโซม ไมโครอิมัลชัน และนาโนอิมัลชันทุกตำรับ ยกเว้น NE2) เป็นเวลาอย่างน้อย 8 สัปดาห์ ระบบนาโนทุกตำรับมีการปลดปล่อยสารสกัดออกมาอย่างช้า ๆ จากการศึกษาการซึมผ่านผิวหนังแบบนอกกายที่ใช้ผิวหนังของลูกหมูแรกเกิด พบว่า ระบบนาโนทั้งหมดสามารถนำส่งสารสกัดได้ดีกว่ากลุ่มควบคุม ระบบนาโนอิมัลชันและไมโครอิมัลชันมีประสิทธิภาพในการนำส่งสารสกัดทางผิวหนังได้ดีกว่าระบบเวสซิ-เคิล ในระบบนาโนชนิดต่าง ๆ นาโนอิมัลชันเป็นระบบที่เหมาะสมที่สุดสำหรับนำส่งสารสกัดผลมะขามป้อมเข้าสู่ผิวหนังและผ่านผิวหนัง ดังนั้น นาโนอิมัลชัน (NE1) ไมโครอิมัลชัน (ME3) และอิลาสติกลิโพโซม จึงเป็นระบบที่คัดเลือกเพื่อการพัฒนาเป็นผลิตภัณฑ์เครื่องสำอางต่อไป

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5376856133 : MAJOR PHARMACEUTICAL TECHNOLOGY

KEYWORDS: *PHYLLANTHUS EMBLICA*/SKIN PERMEATION/ANTIOXIDANT/ANTI-TYROSINASE/LIPOSOME/NIOSOME/MICROEMULSION/NANOEMULSION

SETINEE CHANPIROM: *IN VITRO* COMPARATIVE EVALUATION OF VARIOUS NANOSYSTEMS CONTAINING *PHYLLANTHUS EMBLICA* FRUIT EXTRACT. ADVISOR: DUSADEE CHARNVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., ANYARPORN TANSIRIKONGKOL, Ph.D., 147 pp.

Phyllanthus emblica (Ma-Kham Pom) fruit extract is a well known antioxidant and skin lightening agent. Various extraction methods could render extracts with different biological activities. Thus, the present study aimed to find the most active extract of *P. emblica* fruit and to propose the most suitable system for skin delivery. Biological activities and total phenolic content were used to select the most active extract prepared by various extraction methods. The chosen active extract was then incorporated into various nanosystems including conventional liposomes, elastic liposomes, niosomes, microemulsions (ME1, ME2, ME3) and nanoemulsions (NE1, NE2, NE3). Nanosystems were evaluated in terms of appearance, particle size and entrapment efficiency. Their stability, *in vitro* release and *in vitro* skin permeation were performed. Results showed that the ethanolic extract of dried *P. emblica* fruit exhibited high antioxidant activity (IC_{50} = 1.221 ± 0.005 µg/mL), tyrosinase inhibition activity (IC_{50} = 0.519 ± 0.007 mg/mL) and total phenolic content (490.756 ± 0.185 mg GAE/g of the extract) with high yield. The extract-loaded nanosystems were physically and biologically stable at 4 ºC (for liposomes) or at room temperature (for niosomes, all microemulsions and nanoemulsions except NE2) for at least 8 weeks. Slow releases were observed in all nanosystems. From *in vitro* skin permeation study using newborn pig skin, all nanosystems could deliver the extract better than their controls. Nanoemulsions and microemulsions were more effective in skin delivery of the extract than the vesicular systems. Amongst the nanosystems, nanoemulsions was the most suitable system for dermal and transdermal delivery of the *P. emblica* fruit extract. Therefore, nanoemulsions (NE1), microemulsions (ME3) and elastic liposomes were chosen for further development of cosmetic products.

Department : Pharmaceutics and Industrial Pharmacy Student's Signature .....

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Academic Year : 2012 Co-advisor's Signature .....

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ACKNOWLEDGEMENTS

First of all, I sincerely acknowledge my deep sense of appreciation to my major advisor, Dr. Dusadee Charnvanich. My thesis would not be fully accomplished without her invaluable advice, encouragement and support. I would like to give a very thankfulness to my co-advisors, Associate Professor Dr. Waraporn Suwakul and Dr. Anyarporn Tansirikongkol to their kindness and valuable guidance during the entire duration of work. I would like to thank Assistant Professor Dr. Boonsri Ongpipattanakul for her advice and opportunity for research funding support.

Beside, I would like to thank the thesis committee for their constructive suggestions and invaluable comments. I am the most grateful for depth review that precede the completion of this thesis.

Thanks are extended to Mae Fah Luang University for financial support. I also would like to thank the National Nanotechnology Center (NANOTEC), Thailand for research funding support (Grant number P-00-10644), instruments and facilities. Kindly thanks to Chulalongkorn University Drug and Health Products Innovation Promotion Center (CU.D.HIP) for facility support.

I would like to acknowledge to Associate Professor Dr. Ubonthip Nimmannit, Dr. Apirada Sucontphunt and her colleagues at NANOTEC for their assistance and great support. I thank all staff members of the Pharmaceutical Research Instrument Center, and Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University for laboratory facilities.

I thank to Soontorn Farm for supporting newborn pigs used in permeation study.

Furthermore, I would like to the deepest gratitude to my family and friends for their love, understanding and encouragement throughout this thesis.

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LIST OF ABBREVIATIONS

%	percentage
ºC	degree Celsius
µg	microgram (s)
µL	microliter (s)
µm	micrometer (s)
ANOVA	analysis of variance
cm^2	square centimeter
CV	coefficient of variation
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EF	enhancement factor
FT	freeze-thaw cycle
g	gram (s)
GAE	gallic acid equivalents
HC	heating-cooling cycle
HPLC	high pressure liquid chromatography
hr	hour (s)
IC_{50}	half maximal inhibitory concentration
J_{ss}	steady state flux
kDa	kilodalton
L	liter (s)
L-DOPA	L-Dopachrome
mbar	millibar
ME	microemulsions
mg	milligram (s)
min	minute (s)
mL	milliliter (s)
mM	millimolar (s)
NaOH	sodium hydroxide
N/A	not available
ND	not determined
NE	nanoemulsions

nm	nanometer (s)
no.	number
PBS	phosphate buffer saline
PDI	polydipersity index
P. emblica	*Phyllantus emblica*
PG	propylene glycol
P_s	permeability coefficient
Q_s	amount of extract accumulated in skin per diffusion area at 24 hr
Q_{24}	cumulative amount of permeated extract per diffusion area at 24 hr
r	coefficient of correlation
r^2	coefficient of determination
RF	relative flux
rpm	revolution per minute
RT	room temperature
SD	standard deviation
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

CHAPTER I

INTRODUCTION

Phyllanthus emblica, commonly known in Thailand as Ma-Kham Pom, belongs to Euphorbiaceae. It grows naturally in tropical and subtropical parts of Thailand. *P. emblica* is of interest as an antioxidant and a skin lightening agent for cosmetics. It has been reported that *P. emblica* fruit extract contains ascorbic acid (Zhang et al., 2001; Majeed et al., 2008), tannins, benzenoids and flavonoids (Summanen, 1999). Different extraction methods and solvents could render extracts with dissimilar biological activities and total phenolic contents. Antioxidant, anti-tyrosinase activities and total phenolic content of the *P. emblica* extract extracted from different methods and solvents were reported (Pornpen Kongaimpitee, 2007; Sirirat Pinsuwan et al., 2007; Liu et al., 2008; Boontarika Kawswang et al., 2009; Jantima Homklob et al., 2010). In this study, four extracts of *P. emblica* fruits were used: spray-dried powder of *P. emblica* compressed juice, spray-dried powder of ethanolic extract of fresh fruit, ethanolic extract of dried fruit and ethyl acetate extract of dried fruit. The extracts were evaluated for their antioxidant, anti-tyrosinase activities and total phenolic content in order to select the most active extract for further nano-cosmetic application.

Skin is a target for topical delivery of active compounds to express the antioxidant and whitening effects. A natural barrier of skin called stratum corneum is a major obstacle for percutaneous penetration (Bronaugh and Maibach, 2005). The various polarities of active compounds in *P. emblica* extract also limited its delivery to skin and its high acidity could cause skin irritation. Accordingly, the uses of nanosystems have been introduced to overcome this problem. Many studies showed that nanosystems could improve drug delivery to skin. For examples, liposomes and niosomes increased permeation of dithranol through mouse skin (Agarwal, Katare, and Vyas, 2001). Microemulsions could significantly increase the permeation of penciclovir into both epidermis and dermis (Zhu et al., 2009). Moreover, nanosystems can entrap both lipophilic and hydrophilic drugs, control drug release and increase solubility of drug (Schreier and Bouwstra, 1994; Lawrence and Rees, 2000). From the

advantages mentioned above, nanosystems are interesting and suitable to be used for dermal delivery of *P. emblica* extract.

Liposomes are one of the widely used nanosystems. They can deliver both hydrophilic and hydrophobic drugs. Hydrophilic drugs are entrapped in the aqueous core and lipophilic drugs are associated with lipid membrane (New, 1990). There are a number of reports on the successful skin delivery of many compounds by liposomes such as triamcinolone acetonide (Yu and Liao, 1996), oestradiol (El Maghraby, Williams, and Barry, 1999), glycolic acid (Perugini et al., 2000) and enoxacin (Fang et al., 2001). Liposomes of *P. emblica* extract also were reported. Pornpen Kongaimpitee (2007) and Ubonthip Nimmannit et al. (2009) prepared liposomes containing 0.25 %w/v of the spray-dried *P. emblica* juice extract by film hydration method. The liposomes were composed of egg phosphatidylcholine and cholesterol. The result showed that liposomes serum did not show significant improvement in skin lightening on the volunteers. This result may be because of low effectiveness of the extract and low entrapment efficiency as well as ineffectiveness of the conventional liposomes for dermal delivery of the extract.

To solve the problem, the most active extract was chosen regarding to its antioxidant, anti-tyrosinase activities and total phenolic content. The maximum extract loading into liposomes was determined as the saturated concentration of the extract. At saturation, the highest thermodynamic activity is occurred and the maximum flux of a drug across a membrane is obtained (Higuchi, 1960). Low entrapment efficiency of the extract in liposomes was overcome by preparation methods. Preparation methods of liposomes affect the entrapment efficiency of drugs in liposomes (Szoka, 1980; Riaz, 1996; Maestrelli et al., 2006; Setha and Misra, 2007). Film hydration is a widely used method for liposome preparation. However, it is a multi-step process and yields liposomes with heterogeneous size distribution. Another method used in this study was microfluidization. It is one of liposome preparation techniques for large scale manufacture. Its main advantage includes continuous production of large-scale batch with narrow size distribution (Pegg and Shahidi, 2007). A high entrapment efficiency could be obtained for liposomes containing pirarubicin (Cong et al., 2010) when they was prepared using microfluidization. Moreover, microfluidization can improve the

entrapment efficiency of liposomes containing hemoglobin (Zheng et al., 1999) compared to reverse phase evaporation and film hydration method. In this study, thus, film hydration and microfluidization techniques were investigated on entrapment efficiency of the extract in conventional liposomes.

Since conventional liposomes may not be suitable for dermal delivery of the extract, elastic liposomes, niosomes, microemulsions and nanoemulsions are alternative systems. Elastic liposomes are similar to conventional liposomes but with the addition of an edge activator, such as sodium cholate, Span® 80 and Tween® 80, providing elasticity (Bouwstra et al., 2002; Honeywell-Nguyen et al., 2004). Elastic liposomes are claimed to improve skin delivery of many compounds compared to conventional liposomes such as dexamethasone (Jain et al., 2003), diclofenac (Boinpally et al., 2003) and melatonin (Dubey et al., 2006). Elastic liposomes could squeeze through tiny channels and spontaneously penetrate the stratum corneum and deeper epidermis (Cevc, 1996). Elastic liposomes containing the ethanolic *P. emblica* dried fruit extract were reported by Sirirat Pinsuwan et al. (2007). It was prepared from soybean phosphatidylcholine, Tween® 80 and deoxycholic acid by ethanol injection. The result showed that *in vitro* skin permeability of the extract from liposomes was significantly higher than its solution.

Niosomes are non-ionic surfactant-based vesicles whose structure is similar to that of liposomes. Non-ionic surfactant is used to form vesicles instead of phospholipid to provide lower cost, greater stability and ease of storage (Uchegbu and Vyas, 1998; Choi and Maibach, 2005). Many compounds were successfully incorporated into and transported by niosomes such as carboxyfluorescein (Yoshioka, Sternbergb, and Florence, 1994), estradiol (Hofland et al., 1994), retinoic acid (Montenegro, Ventimiglia, and Bonina, 1996), propylthiouracil (Waraporn Suwakul, 2005) and N-acetyl glucosamine (Shatalebi, Mostafavi, and Moghaddas, 2010). The *P. emblica* extract loaded niosomes composed of Span® 20 and cholesterol were prepared by ethanol injection (Boontarika Kawswang et al., 2009). The report showed an increase in skin permeation of the extract and skin whitening improvement in volunteers.

Microemulsions are thermodynamically stable dispersed systems of oil and water stabilized by surfactant with or without cosurfactant. Microemulsions are formed spontaneously. Microemulsions are transparent, low viscous and small particle size of less than 100–200 nm. The main advantage of microemulsions is their high solubilization capacity for various compounds. In this study, microemulsions are expected to increase solubility of *P. emblica* extract. Other advantages of microemulsions are penetration enhancement, low energy input in preparation, as well as, good physical stability over a wide temperature range (Date, Naik, and Nagarsenker, 2006; Santos et al., 2008; Lu, and Gao, 2010). The potential in cutaneous drug delivery by microemulsions was demonstrated in many researches such as triptolide (Chen et al., 2004), ibuprofen (Chen et al., 2006), meloxicam (Yuan et al., 2006), *Aloe vera* and *Arnica montana* components (Bergamante et al., 2007), penciclovir (Zhu et al., 2009) and silymarin (Panapisal, Charoensri, and Tantituvanont, 2012).

Nanoemulsions are non-equilibrium dispersed systems of submicron droplets of oil and water. Nanoemulsions are generally formulated through high-energy methods or low-energy methods (Anton, Benoit, and Saulnier, 2008). Nanoemulsions improve solubility of compounds while surfactant concentration is lower than microemulsions. Dermal and transdermal delivery enhancement of many drugs by nanoemulsions were published such as aceclofenac (Shakeel, Baboota, and Shafig, 2007), carvedilol (Dixit, Kohli, and Baboota, 2008), celecoxib (Shakeel et al., 2008), ketoprofen (Kim et al., 2008) and coenzyme Q10 (Junyaprasert et al., 2009).

The present study has been aimed to improve efficacy of cosmetic product of *P. emblica* fruit extract by using the most active extract, liposomes with high entrapment efficiency and alternative nanosystems. The most active extract was selected by evaluating biological activities (antioxidant and antityrosinase) and total phenolic content of *P. emblica* fruit extracts prepared using different extraction methods. The effect of preparation methods (film hydration and microfluidization) on entrapment efficiency was studied to increase entrapment efficiency of conventional liposomes. In addition, various nanosystems including elastic liposomes, niosomes, microemulsions and nanoemulsions were investigated as alternative carriers. Physicochemical properties, stability, *in vitro* release and *in vitro* skin permeation of each nanosystem

were investigated and compared. The results will be helpful to provide nanosystems suitable for further application in cosmetic industry.

The purposes of this study were as follows:

1. To compare antioxidant and anti-tyrosinase activities and total phenolic content of *P. emblica* fruit extract prepared using different extraction solvents and drying methods
2. To investigate effect of preparation methods on entrapment efficiency of *P. emblica* fruit extract in conventional liposomes
3. To investigate stability of nanosystems containing *P. emblica* fruit extract
4. To compare *in vitro* release and *in vitro* skin permeation of *P. emblica* fruit extract from different nanosystems

CHAPTER II

LITERATURE REVIEW

Phyllanthus emblica

Phyllanthus emblica is well known as 'Ma-Kham-Pom' in Thai. It is in Euphorbiaceae family. It grows naturally in tropical and subtropical parts of Thailand, China, India, Indonesia, and Malaysia. *Phyllanthus emblica* is a small to medium plant with thin light grey bark, pinnate leaves and greenish yellow flowers. The fruits are globule, fleshy, pale yellow with vertical furrows enclosing six trigonous seeds.

According to the literatures, *P. emblica* contains ascorbic acid, alkaloid, benzenoid, flavonoids, sterols, tannins, and triterpene. These substances are claimed for antioxidant, skin lightening agent, anti-inflammatory, antipyretic, anti-atherosclerotic, antidiabetic, antimutagenic and anticancer (Summanen, 1999; Zhang et al., 2001; Majeed et al., 2008).

In cosmetics, the antioxidant and skin lightening properties of *P. emblica* extract are interesting. The different extraction methods and the solvent used in the isolation of compounds could render extracts with dissimilar chemical substances which gave different properties. Many researchers determined the biological activities and total phenolic content of *P. emblica* especially fruit extract. The spray-dried extract of *P. emblica* compressed juice without any purification showed total phenolic content about 320 and 308 mg GAE/g in buffer solution pH 5.5 and pH 7.4, respectively (Pornpen Kongaimpitee, 2007). The ethanolic extract of dried *P. emblica* fruit prepared using maceration exhibited high antioxidant effect with IC_{50} of 1.55 ± 0.35 μg/mL in DPPH assay and total phenolic content of 454.7 ± 27.0 mg GAE/g (Sirirat Pinsuwan et al., 2007). Using the same extraction method with methanol, the study of Liu et al. (2008) showed lower DPPH scavenging activity (IC_{50} of 11.23 - 45.44 μg/mL) than that of Sirirat Pinsuwan et al. (2007). In the study of Boontarika Kawswang et al. (2009), the ethanolic extract of fresh *P. emblica* fruit was prepared using percolation method. Its total phenolic content (3 mg GAE/g) was lower than that in the study of Sirirat

Pinsuwan et al. (2007), who used the same solvent but different extraction method. In addition, the different sources of raw materials also affected the properties of the extract. Jantima Homklob et al. (2010) determined the ethyl acetate extracts of dried *P. emblica* fruit from different regions in Thailand prepared using soxhlet extraction. Their free radical scavenging capacity, tyrosinase inhibition activity and total phenolic content were in ranges of IC_{50} of 0.025 - 0.037 mg/mL, IC_{50} of 0.151- 0.710 mg/mL and 345 - 597 mg GAE/g, respectively.

Antioxidant properties of *P. emblica* extract on cell culture study also were reported. Fujii et al. (2008) revealed *P. emblica* extract exhibited the matrix metalloproteinase-1 (MMP-1) inhibition and promotion of pro-collagen production in human skin fibroblasts. Recently, Adila et al. (2010) studied the effect of ethanolic extract of *P. emblica* against UVB-induced photo-aging in human skin fibroblasts. The study concluded that the extract could inhibit UVB-induced cellular proliferation and protect pro-collagen 1 (10 - 40 μg/mL). This effect caused by inhibition of UVB-induced MMP-1.

Although *P. emblica* extract provides many valuable active constituents, their stability is a problem. นิริน อุดมสม, กชกร สินสืบผล และนนทยา นวศีลวัตร์ (2548) studied the effects of temperature and pH on stability of *P. emblica* extract. They found that the polyphenol content did not significantly change after storage at room temperature and 40 ºC for 4 weeks. However, it slightly decreased after storage at 60 ºC for 4 weeks. They also found that the polyphenol content of *P. emblica* extract was the most stable during storage at pH 5.5 compared to pH 3.5, 7.4 and 9. The similar result was investigated by Pornpen Kongaimpitee (2007) who found that stability of *P. emblica* extract investigating by total phenolic content was better in buffer solution pH 5.5 than in buffer solution pH 7.4. From these results, it may be concluded that *P. emblica* extract is the most chemically stable in buffer solution pH 5.5 and at storage temperature below 60 ºC.

Beside the stability problem, *P. emblica* extract contains both hydrophilic and hydrophobic compounds which are difficult to transport into skin. Moreover, high

acidity of *P. emblica* extract can cause skin irritation. Thus, nanosystems were introduced.

Nanosystems as delivery systems

The target of topical delivery is skin where the active compounds can express their effects. In this study, the antioxidant and skin lightening agents were focused. As an antioxidant, a drug has to defense against free radicals in skin cells and permeate deeper into dermis where collagen, fibroblast and elastic fiber are produced. To lighten the skin, the epidermis is a target site. The melanin which is responsible for pigmentation of the skin is synthesized and distributed in this layer. Unfortunately, a natural skin barrier called stratum corneum is a major obstacle for percutaneous penetration (Bronaugh and Maibach, 2005). During the past decades, there is an attempt to find new methods to overcome this barrier and increase the permeation of drugs to or through skin. One of these methods is the use of nanosystems such as liposomes, niosomes, microemulsions and nanoemulsions. Many studies showed that nanosystems could improve drug delivery to skin.

1. Conventional liposomes

Liposomes are the generally used nanosystems to deliver both hydrophilic and hydrophobic drugs. They are phospholipid-based vesicles which hydrophilic drugs are entrapped in the aqueous core and lipophilic drugs are associated with lipid membrane. Liposomes are considered to transport drugs via the different possible mechanisms which have been suggested as follows: 1) the release of drug from the vesicle and permeation into and through the skin; 2) the penetration enhancing properties of liposome components which disrupt the structure of stratum corneum to enter the skin; 3) the adsorption of vesicles to and fusion with stratum corneum; 4) the increasing of skin partitioning of drug 5) the penetration through transappendageal pathway (New, 1990).

Liposomes are widely used to deliver several drugs through skin. For example, *in vitro* permeation study of Yu and Liao (1996) showed that flux and permeability of triamcinolone acetonide liposomes were significantly higher than those

of a commercial ointment. El Maghraby et al. (1999) studied *in vitro* permeation of oestradiol through human skin. They revealed that lipid vesicles improved the skin delivery of oestradiol compared with delivery from an aqueous control. Perugini et al. (2000) investigated the feasibility of topical controlled delivery of liposomes, liposomes modified by chitosan addition and chitosan microspheres loading glycolic acid. The *in vitro* dissolution study showed that liposomes were suitable for modulating the release of glycolic acid.

To deliver *P. emblica* fruit extract through the skin, liposomes have been used. Pornpen Kongaimpitee (2007) and Ubonthip Nimmannit et al. (2009) prepared liposomes containing egg phosphatidylcholine, cholesterol and cholesterol ester by film hydration method. Phosphate-citrate buffer solution pH 5.5 was used as a hydration medium. The 0.25 %w/v of the spry-dried *P. emblica* juice extract was incorporated. Unfortunately, serum of these liposomes did not show significant improvement in skin lightening on the volunteers.

2. Elastic liposomes

Elastic liposomes (deformable liposomes or Transfersomes®) are the liposomes having surfactants, such as sodium cholate, Span® 80 and Tween® 80. These surfactants act as edge activators which impart the elasticity of the lipid bilayer (Bouwstra et al., 2002). The penetration of elastic liposomes through the intact stratum corneum is under the effect of transepidermal osmotic gradient. The difference in water concentrations between the surface and interior of skin drives drug penetration to skin. Accordingly, osmotic gradient can be increased under non-occlusive condition. The flexibility of elastic liposomes facilitate them to penetrate through the stratum corneum (Cevc, 1996) and then spontaneously permeate through the deeper skin. Elastic liposomes were reported to transport many drugs through skin. El Maghraby et al. (1999) investigated skin delivery of oestradiol by the elastic vesicles comprising phosphatidylcholine with Tween® 80, Span® 80 or sodium cholate. They found that the deformable liposomes could deliver oestradiol across human skin and improved fluxes of oestradiol over those of conventional liposomes. The similar result was also reported by Trotta et al. (2004) who investigated the penetration across excised pig skin of

methotrexate from elastic liposomes. They found that the penetration of methotrexate increased threefold compared with an aqueous solution and conventional liposomes. Other investigators also proved the better skin delivery of various drugs by elastic liposomes compared to conventional liposomes, for examples, dexamethasone (Jain et al., 2003), diclofenac (Boinpally et al., 2003) and melatonin (Dubey et al., 2006). In addition, *in vivo* study also has been reported. Guo et al. (2000) determined the effectiveness of elastic vesicles composed of lecithin and sodium cholate on the penetration of cyclosporin A. The study showed that with the application of elastic vesicles on the abdominal skin of mice, cyclosporin A could be transported into the blood circulation after 2 hr of application while conventional vesicles failed.

The skin permeability of elastic liposomes is influenced by many factors. One of these is types of edge activators. Jain et al. (2003) reported that the permeation of dexamethasone from Span® 80 elastic liposomes was higher than that from sodium deoxycholate and Tween® 80 elastic liposomes, respectively. El Zaafarany et al. (2010) reported that the permeation of diclofenac sodium was higher from Tween® 80 elastic liposomes than sodium deoxycholate, sodium cholate, Span® 80 and Span® 85 elastic liposomes. However, El Maghraby et al. (1999) reported that *in vitro* permeation of oestradiol from Span® 80, sodium deoxycholate and Tween® 80 elastic liposomes was not significantly different.

For delivery of *P. emblica* extract, the use of elastic liposomes was studied. Sirirat Pinsuwan et al. (2007) prepared elastic liposomes composed of soybean phosphatidylcholine, Tween® 80 and deoxycholic acid by injection method. The 1 %w/w ethanolic extract of dried *P. emblica* fruit was incorporated. The result showed the permeation improvement of the *P. emblica* extract compared with the emblica extract solution.

3. Niosomes

Niosomes are self-assembled vesicles formed by non-ionic surfactant together with cholesterol. Because of low cost production, better chemical and physical stability, and ease of storage, they are an alternative carrier to liposomes (Uchegbu and

Vyas, 1998; Choi and Maibach, 2005). The penetration mechanisms of niosomes are similar to liposomes.

Niosomes were claimed to transport many drugs through skin, for examples, *in vitro* permeation and *in vivo* deposition studies of Tabbakhian et al. (2006) demonstrated that niosomes were successful to deliver finasteride to the pilosebaceous unit. Shatalebi et al. (2010) showed that the extent of N-acetyl glucosamine localized in the skin was significantly improved by niosomes in comparison with hydroalcoholic solution. Junyaprasert et al. (2012) revealed that niosomes with Span® 60 or Tween® 60 enhanced the permeation of ellagic acid, which has low solubility and poor biopharmaceutical property. These studies demonstrated the potential of niosomes as the skin delivery system. Delivery of other drugs by niosomes were also reported such as carboxyfluorescein (Yoshioka et al, 1994), diclofenac sodium (Naresh et al., 1994), estradiol (Hofland et al., 1994), 5-retinoic acid (Montenegro et al., 1996) and propylthiouracil (Waraporn Suwakul, 2005).

Compared to liposomes, Fang et al. (2001) showed the superior transdermal delivery of enoxacin from niosomes than that from liposomes. On the other hand, Agarwal et al. (2001) reported that skin permeation of dithranol from liposomes was higher than that from niosomes. From these previous studies, the skin permeability of drugs from niosomes compared to liposomes seemed to be unclear depending on drug properties and vesicle compositions.

The effect of surfactant in niosomes on permeation of drugs is evident. Yoshioka et al. (1994) concluded that Span® 20 and Span® 80 niosomes of carboxyfluorescein showed higher drug permeation than Span® 40 and Span® 60 niosomes. Balakrishnan et al. (2009) reported that *in vitro* permeation of minoxidil from Span® 20 and Span® 40 niosomes was higher than Span® 60 niosomes. The thermodynamic state mainly contributed to these results. The gel state niosomes (Span® 40 and Span® 60 niosomes) gave lower skin permeation than the liquid crystalline niosomes (Span® 20 and Span® 80 niosomes).

There was a study investigating on niosomes containing *P. emblica* extract. Boontarika Kawswang et al. (2009) studied the release of ethyl acetate extract of fresh *P. emblica* fruit (1 %w/w) from Span® 20 niosomes. The result showed insignificant difference between that of niosomes and its solution. However, the permeation of the *P. emblica* extract from niosomes was better than that of its solution and cream containing niosomes of *P. emblica* extract could also significantly improve skin whitening in volunteers.

4. Microemulsions

Microemulsions are thermodynamically stable dispersed systems of oil, water, surfactant and co-surfactant. A high proportion of surfactant and cosurfactant is used to lower surface tension. Unlike to emulsions, microemulsions are transparent and spontaneously formed (Lawrence and Rees, 2000). The skin permeation of drugs by microemulsions is possibly the result of their component effects. Surfactant, co-surfactant and some oils, such as oleic acid and isopropyl myristate, were reported as penetration enhancer (Karande and Mitragotri, 2009). The surfactants can interfere or solubilize the intercellular lipid structure of skin and allow drugs pass through skin (Heuschkel, Goebel, and Neubert, 2008). The oil phase may provide the occlusion effects and then increase skin hydration (Amselem and Friedman, 1998). Furthermore, the solubility of drugs in microemulsions is related to skin delivery rate. The higher solubility of drugs in microemulsions gives the higher concentration gradient of the drugs between skin surface and inside the skin

The potential in cutaneous drug delivery by microemulsions was demonstrated. Chen et al. (2004) loaded triptolide into microemulsions composed of oleic acid, Tween® 80, propylene glycol and water. The *in vitro* permeation through mouse skin showed that microemulsions enhanced the permeation of triptolide compared to an aqueous solution of 20 % propylene glycol. Chen et al. (2006) studied the *in vitro* permeation of the developed microemulsions for delivery of ibuprofen. The results showed that microemulsions comprising ethyl oleate, Tween® 80, propylene glycol and water could increase the permeation rate of ibuprofen 5.72-30.0 folds over the saturated solution. Meloxicam from microemulsions composed of isopropyl

myristate, Tween® 85, ethanol and water was permeated through skin from *in vitro* study (Yuan et al., 2006). The uses of microemulsions as delivery systems also were reported for *Aloe vera* and *Arnica montana* components (Bergamante et al., 2007), penciclovir (Zhu et al., 2009) and silymarin (Panapisal et al., 2012).

Microemulsions were found to result higher skin penetration than macroemulsions for betamethasone valerate and dipropionate, indomethacin, diclofenac, piroxicam, and naproxen (Friedman, Schwarz, and Weisspapir, 1995). In contrast, a comparative study of macroemulsions and microemulsions found no significant difference in the skin penetration of tetracaine (Izquierdo et al., 2007).

5. Nanoemulsions

Nanoemulsions are non-equilibrium dispersed systems of submicron droplets of oil and water. Nanoemulsions are generally formulated through high-energy methods or low-energy methods (Anton et al, 2008). Nanoemulsions improve solubility of compounds while surfactant concentration is lower than microemulsions. The skin permeation of drug by nanoemulsions was explained. Mou et al. (2008) developed a hydrogel-thickened nanoemulsions of lipophilic drugs such as camphor, menthol and methyl salicylate. It was found that the high permeation rates of the nanoemulsions might be attributed to the high concentration gradient due to the high concentration of drugs. They also stated that nanoemulsions acted as reservoirs for the release of an active compound. Several compounds used in nanoemulsions act as penetration enhancers and were reported to improve the transdermal permeation by altering the structure of the stratum corneum (Karande and Mitragotri, 2009). Shakeel et al. (2008) indicated that permeation of celecoxib by nanoemulsions was due to the extraction of lipids in stratum corneum.

Many studies showed that nanoemulsion formulations had a potential for transdermal and dermal delivery. Shakeel et al. (2007) revealed the potential of nanoemulsions on the *in vitro* skin permeation of aceclofenac. The result showed that steady-state flux, permeability coefficient, and enhancement ratio of the optimized nanoemulsions were higher compared to those of conventional gel. The similar result

was also found in carvedilol (Dixit et al., 2008), of which the steady-state flux and permeability coefficient significantly increased when observed in nanoemulsions. The higher permeation was obtained compared to drug-loaded neat components. Ketoprofen loaded nanoemulsions could enhance *in vitro* permeation through mouse skin compared to ethanol solution (Kim et al., 2008). Nanoemulsion gel improved transdermal permeation of ropinirole over the conventional hydrogel (Azeem et al., 2009). The similar result was also found for celecoxib (Baboota et al., 2007). The *in vitro* skin permeation of celecoxib nanoemulsion gel was investigated in comparison with celecoxib gel. A significant increase in the steady state flux, permeability coefficient and enhancement ratio was observed in nanoemulsion formulations.

Preparation methods of liposomes

There are various methods to produce liposomes such as film hydration, freeze-drying, ethanol injection, reverse-phase evaporation, etc. (New, 1990). Different preparation methods of liposomes were reported to affect the entrapment efficiency (Szoka, 1980; Riaz, 1996; Maestrelli et al., 2006; Seth and Misra, 2007). To improve the entrapment efficiency, the effective method should be investigated. The following is a brief description of methods that are considered in this study.

1. **Film Hydration Method** (New, 1990; Riaz, 1996)

Film hydration is the most simple and widely used method for liposome preparation. Briefly, the mixture of lipid in a suitable organic solvent, such as methanol or chloroform, is removed under reduced pressure to obtain a thin film at the bottom of round bottom flask. The thin lipid film is then rehydrated with an aqueous medium at a temperature above the gel-liquid crystalline transition temperature of lipids. The drugs are added either in aqueous medium for hydrophilic drugs or to organic solvent containing lipids for lipophilic drugs. At the end of this process, the large heterogeneous liposomes are obtained. Further process like sonication or extrusion may be necessary to produce smaller and more uniform liposomes. Moreover, it is not suitable for large-batch manufacturing. In comparison with reverse phase evaporation, acyclovir liposomes prepared by film hydration gave slightly higher entrapment efficiency (Seth

and Misra, 2007). In the study of Maestrelli et al. (2006), encapsulation efficiency of vesicles prepared by film hydration was 42-75% depending on the concentrations of ketoprofen-2-hydroxypropyl-β-cyclodextrin complex. At the same concentration, entrapment efficiency of liposomes prepared by film hydration was higher than reverse phase evaporation method and the extrusion technique. However, New (1990) stated that using film hydration technique, lipid soluble drugs can entrap as high as 100% whereas hydrophilic compounds are often encapsulated in amount of 5-10%.

2. Microfluidization (Riaz, 1996; Muller and Peters, 1998; Maa and Hsu, 1999; Jafari, He and Bhandari, 2007a; Jafari, He and Bhandari, 2007b)

Microfluidization is a new technology for production of liposomes, niosomes and nanoemulsions. Microfluidization flow diagram is pictured in Figure 1. In the process, the formulation compositions in a reservoir is pumped under pressure and flowed to an interaction chamber with very high velocity. In the interaction chamber, two streams of the formulation from two opposite channels are collided with one another. At this point, the droplets are disrupted and reformed with a high shearing. High energy input (high pressure) and reformed droplets (number of cycles) are inevitable, therefore, an appropriateness of them should be used in order to reduce “over-processing”.

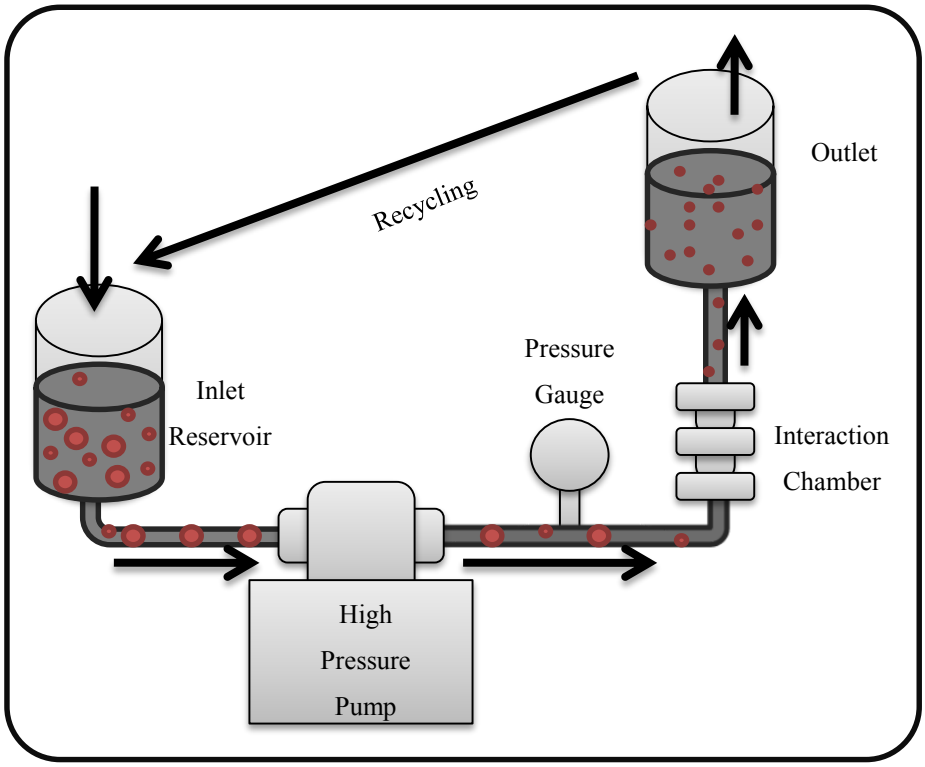


Figure 1 Microfluidization flow diagram (adapted from Horiba Scientific, 2010)

There are a number of papers in the literature reporting a successfulness of microfluidization for production of liposomes (Bae et al, 2009; Cong et al., 2010). With microfluidization, small and uniform liposomes were obtained. This technique is responsible for reproducible large-scale production with less time process.

Moreover, it was reported that microfluidization could provide high entrapment efficiency of liposomes. During microfluidization process, larger liposomes were cut down and rearranged in the interaction chamber. Using microfluidization, high entrapment efficiency (> 93.1 %) could be obtained for liposomes containing pirarubicin (Cong et al., 2010). Zheng et al. (1999) showed that the encapsulated hemoglobin amount in liposomes prepared by microfluidization was higher than that of reverse phase evaporation and film hydration method, respectively. These studies implied that microfluidization was an interesting method to improve the entrapment efficiency of liposomes.

CHAPTER III

METERIALS AND METHODS

Materials

1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Lot no. STBB0447L9, Sigma-Aldrich, USA)
2. Absolute ethanol, AR grade (Lot no. 10100335, Labscan Asia Co., Ltd.,Thailand)
3. Ascorbic acid (Lot no. 327066/1, Fluka, USA)
4. Caprylic/capric triglyceride (Lot no. 17857, Croda, UK)
5. Chloresterol (Lot no. BCBF1727V, Sigma, USA)
6. Chloroform, AR grade (Lot no. 12030118, Labscan Asia Co., Ltd.,Thailand)
7. Cremophor® EL (Lot no. 02584324U0, BASF, Germany)
8. Dicetyl phosphate (Lot no. 076K1579, Sigma-Aldrich, USA)
9. Disodium hydrogen phosphate (Lot no. 91706110E , Carbo Erba Reagenti, Italy)
10. Ethanol, 95% (Lot no. 66005, The Government Pharmaceutical Organization, Thailand)
11. Ethyl oleate (Lot no. 256950, Croda, UK)
12. Follin-Ciocalteu reagent (Merck, Germany)
13. Gallic acid (Lot no. 392513/1, Fluka, USA)
14. Isopropyl myristate (Lot no. 12390H, S.Tong Chemical Co., Ltd, Thailand)
15. Kojic acid (Lot no. 097K2572, Sigma, USA)
16. L-Dopa (Lot no. 129K1885, Sigma-Aldrich, USA)
17. Methanol, AR grade (Lot no. 12050023 Labscan Asia Co., Ltd.,Thailand)
18. Methanol, HPLC grade (Lot no. 10071753 , Burdick&Jackson, USA)
19. Mushroom tyrosinase (T-3824) (Lot no.010M7012, Sigma-Aldrich, USA)
20. Oleic acid (Lot no. 0000334096, Panreac)
21. PEG-8 caprylic/capric glycerides (L.A.S.®, Lot no. 122787, Gattefosse, France)
22. Polyglyceryl-3 Methylglucose Distearate (Tego® Care 450, Lot no. 50107375, Evonik Industries, Germany)
23. Propylene glycol (Lot no. 66352266091, S.Tong Chemical Co., Ltd, Thailand)

24. Sodium carbonate (Lot no. AF405220 , Ajax Finechem, Australia)
25. Sodium citrate (Lot no. 107K0152, Sigma-Aldrich, USA)
26. Sodium dihydrogen phosphate (Lot no. AF502342, Ajax Finechem, Australia)
27. Sodium hydroxide (Lot no. B0200898 , Merck, Germany)
28. Solulan® 75 (Lot no. CR9060100 , Lubrizol Advanced Materials, Inc., USA)
29. Soybean oil (Thai Vegetable Oil Public co, Ltd, Thailand)
30. Soybean phosphatidylcholine (Epikuron® 200, Lot no. 129020, Cargill Inc., USA)
31. Span® 20 (Namsiang Trading Co., Ltd., Thailand)
32. Span® 80 (Namsiang Trading Co., Ltd., Thailand)
33. Trichlorofluoric acid (Lot no. 1393453, Fluka, USA)
34. Tween® 20 (Lot no. 709557, Srichand United Dispensary Co., Ltd., Thailand)
35. Tween® 80 (Lot no. 23601, Croda, UK)
36. Tween® 85 (Lot no. 17754, Croda, UK)

Equipment

1. Analytical balance (GMPH, Sartorius, Germany)
2. Analytical balance (UMTZ, Mettler Toledo, Switzerland)
3. High Performance Liquid Chromatography System (HPLC) (Waters Alliance e2695, Waters Co., USA)
4. Homogenizer (T25 digital, ULTRA-TURRAX®, IKA®, Malaysia)
5. Hot air Oven (Thermo Scientific, Thermo Fisher Scientific, Inc., USA)
6. HPLC column (ACE C18-AR, Advanced Chromatography Technologies, Ltd, Scotland)
7. Light microscope (Nikon Eclipse E200, Nikon, Japan)
8. Magnetic stirrer (modelBasic M6 CAT, IKA® WERKE, Germany)
9. Membrane dialysis, molecular weight cutoff (MWCO) 12-14 kDa (Lot No. 9587, Cellu Sep® T4, Membrane filtration products, Inc., USA)
10. Microfluidizer (Model M-110P, Microfluidics®, USA)
11. Micropipette (Gilson, France)
12. Microplate reader (SpectraMax® M5, Molecular Devices Co., USA)
13. Modified Franz diffusion cells (Science Service, Thailand)
14. pH meter (Model 420A, Orion research, Inc., USA)

15. Polycarbonate bottles with cap assemblies (Beckman Coulter, Inc., USA)
16. Rotary evaporator (Rotavapor R-215, Buchi, Switzerland)
17. Sonicator (Model TP680DH, Elma, Germany)
18. Ultracentrifugation (L80, Beckman, USA)
19. Vortex mixer (Vortex Genie-2, Scientific Industries, USA)
20. Water bath (Model WB22, Becthai Co., Ltd., Thailand)
21. Zetasizer (Zetasizer ZS, Malvern Instruments, UK)

Methods

From the study of Ubonthip Nimmannit et al. (2009), the conventional liposomes containing the *P. emblica* extract showed insignificant difference in the improvement in skin lightening on the volunteers. This study had an attempt to solve the problem. Various extraction methods were studied to obtain the most active extract of *P. emblica* fruit (Part I). Two preparation methods (film hydration and microfluidization) of conventional liposomes were compared to obtain the better entrapment efficiency (Part III, 3.2). In addition, others nanosystems such as elastic liposomes, niosomes, microemulsions and nanoemulsions were investigated as delivery choices (Part III, 3.3).

Part I Comparative evaluation of *Phyllanthus emblica* fruit extracts using various extraction protocols

P. emblica fruit extracts were studied to find the most active extract for cosmetic application. *P. emblica* fruit extracts used in this study were spray-dried powder of *P. emblica* compressed juice, spray-dried powder of ethanolic extract of fresh fruit, ethanolic extract of dried fruit and ethyl acetate extract of dried fruit. The former two extracts were selected from the study of Ubonthip Nimmannit et al. (2009) because of their good appearance and ease of use. The latter two extracts were extracted using 95% ethanol or ethyl acetate. Both solvents were chosen because they were reported to provide the extracts with high antioxidant and anti-tyrosinase activities (Nantanat Mahattanapokai, 2003; Nadheesha et al., 2007; Boontarika Kawswang et al., 2009).

Because of a limitation in storage and sources of fresh fruit had, dried fruit was also extracted to compare their properties. The extraction was performed as follows. The 100 g of dried *P. emblica* fruit was ground and soaked in 400 mL of 95% ethanol or ethyl acetate for 24 hours, followed by filtration (Whatman no. 1). The residue was re-extracted for 2 times with the same volume of solvent. Then, the filtrate was combined and concentrated by rotary evaporator at 40 °C and left on water bath (45 ºC) until the constant weight was achieved. The extracts were weighed and calculated for their yields as shown in equation (1);

$$\%\text{Yield} = \frac{\text{Weight of an obtained extract}}{\text{Weight of dried } \textit{P.emblica} \text{ fruit}} \times 100\% \quad (1)$$

Extracts were packed in tightly glass containers and placed in a refrigerator until used.

Then, their antioxidant, anti-tyrosinase activities and total phenolic content were evaluated as follows to select the most active extract for further studies.

1.1 DPPH radical scavenging activity

The antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical. Radical scavenging activity of the extract against stable DPPH was determined spectrophotometrically as reported by Miliauskas and Venskutonis (2004) with modifications. Briefly, a portion of the sample solution (100 µL) in absolute ethanol was mixed with 100 µL of 6×10^{-5} M DPPH in absolute ethanol in a 96-well microplate. The mixture was allowed to stand in the dark at room temperature for 30 minutes. When DPPH reacts with an antioxidant, which can donate hydrogen, it is reduced. The changes in color (from violet to light yellow) were measured at 517 nm on a microplate reader. Each assay was performed in triplicate. Ascorbic acid and gallic acid were used as reference compounds. The percent DPPH inhibition of antioxidant activity was calculated as follows:

$$\%\text{DPPH inhibition} = \left(\frac{\text{(A-B)- (C-D)}}{\text{(A-B)}}\right) \times 100\% \quad (2)$$

Where; A is the absorbance at 517 nm without test sample
B is the absorbance at 517 nm without test sample and DPPH solution
C is the absorbance at 517 nm with test sample
D is the absorbance at 517 nm with test sample, but without DPPH solution

The concentration of test sample that can cause 50% inhibition of free radicals (IC_{50}) was obtained by plotting % inhibitions versus concentrations of test sample. Lower IC_{50} value indicates higher antioxidant activity.

1.2 Tyrosinase inhibition activity

Tyrosinase inhibition activity was determined using the modified dopachrome method with L-Dopa as a substrate (Boonchoo Sritularak, 2002). The 40 µL of sample in 60 %w/v PG, 80 µL of 20 mM phosphate buffer (pH 6.8) and 40 µL of mushroom tyrosinase in phosphate buffer pH 6.8 (480 units/mL) were mixed in a 96-well microplate. The mixture was pre-incubated at 25 °C for 10 minutes and then followed by an addition of 20 µL of 0.85 mM L-dopa substrate prior to incubation at 25 ˚C for 10 minutes. The changes in color from colorless to red caused by formation of dopachrome were measured at wavelength of 492 nm using a microplate reader. There was a corresponding blank for each sample, and this blank contained all components except for the mushroom tyrosinase. Gallic acid and kojic acid were used as reference compounds. Each assay was performed in triplicate. The tyrosinase inhibition activity was calculated using equation (3):

$$\%\text{Tyrosinase inhibition} = \left(\frac{(A\text{-}B)\text{-}(C\text{-}D)}{(A\text{-}B)}\right) \times 100\% \qquad (3)$$

Where; A is the absorbance after incubation at 492 nm without test sample
B is the absorbance after incubation at 492 nm without test sample and enzyme
C is the absorbance after incubation at 492 nm with test sample

D is the absorbance after incubation at 492 nm with test sample, but without enzyme

The concentration of test sample that can cause 50% inhibition of dopachrome production (IC_{50}) was obtained by plotting % inhibitions versus concentrations of test sample. Lower IC_{50} value indicates higher tyrosinase inhibition activity.

1.3 Total phenolic content

Total phenolic content of the extract was measured by using a modified Folin-Ciocalteau method (Miliauskas and Venskutonis, 2004). Folin-Ciocalteau reagent is a mixture of phosphomolydate and phosphotungstate which will oxidize phenolates and reduce the heteropoly acids to a blue Mo-W complex (Singleton and Rossi, 1965). The sample solution in deionized water (20 µL) was mixed with 100 µL of the ten-fold diluted Folin–Ciocalteu reagent and 80 µL of sodium carbonate (75 g/L). The mixture was incubated at room temperature for 1 hr. The absorbance of the reaction mixture was measured at 765 nm on a microplate reader. All determinations were performed in triplicate. Gallic acid in the concentrations between 1.2-15 µg/mL was used to prepare a standard curve for quantifying the phenolic contents. The results were expressed as milligram gallic acid equivalent (GAE) per gram of the extract.

The extract that provided the highest DPPH radical scavenging activity, tyrosinase inhibition activity and total phenolic content with suitable % yield was selected for further studies.

Part II Evaluation of physicochemical properties of the selected crude extract

2.1 Quantification of gallic acid content

Gallic acid is a key phenolic compound existing in *P. emblica* fruit which poses antioxidant and anti-tyrosinase activities (Chang, 2009; Majeed et al., 2008). Therefore, gallic acid content in the selected extract was determined by HPLC method. The selected extract was dissolved in 0.03 %v/v trifluoroacetic acid at the concentration of 0.1 mg/mL and the solution was filtered through 0.45 μm syringe filter. Gallic acid content in *P. emblica* fruit extract was detected at 270 nm by HPLC under following conditions (Witsarut Booranasatja, Chanida Kanpracha and Charnnarong Nakjumradsri, 2005):

HPLC instrument	: Water Model Alliance e 2695
Column	: ACE® C18 (250 mm × 4.6 mm, 5μm i.d.)
UV detector	: Photodiode Array Detector Model 2998
Mobile phase	: 0.03 %v/v Trifluoroacetic acid:Methanol (92:8)
Injection volume	: 10 μL
Flow rate	: 1 mL/min
Column temperature	: 30 °C

Gallic acid content in the extract was calculated from a standard curve of gallic acid obtained by plotting concentrations of gallic acid (10, 20, 40, 60 and 80 μg/mL) versus area under curves. It was then expressed as milligram gallic acid per gram of extract. All determinations were performed in triplicate.

2.2 Saturation solubility of the extract in water and ethanol

Solubility of a compound is the fundamental physicochemical property, so solubility of the selected extract was investigated. Estimated solubility of the selected extract was determined in water and ethanol at room temperature. One milligram of the extract was added in 3 mL of a solvent. The mixture was sonicated until clear solution was obtained or for 1 hour. Cloudiness or precipitate was visually observed when its

concentration was above a saturated concentration. If no cloudiness or precipitate was observed, higher concentration was prepared. If cloudiness or precipitate was visually observed, the dilution with the same solvent was performed. The maximum extract concentration giving a transparent solution for 3 days was defined as the saturation solubility of extract in that solvent. All experiments were performed in triplicate.

Part III Comparison of various nanosystems for skin delivery of the selected *P. emblica* fruit extract

In this experiment, conventional liposomes, elastic liposomes, niosomes, microemulsions and nanoemulsions were investigated as choices for skin delivery of the selected extract. Elastic liposomes were used because it has been reported to improve skin delivery compared to conventional liposomes. As lower cost, greater stability and easier in storage, niosomes were chosen as an alternative vesicular system. Moreover, microemulsions were selected because it provided high solubilization capacity, penetration enhancement and good physical stability. However, high surfactant content of microemulsions could cause skin irritation. Thus, a lower surfactant system, nanoemulsions were also chosen.

From preliminary study, conventional liposomes, which were modified from Ubonthip Nimmannit et al. (2009), were composed of soybean phosphatidylcholine, cholesterol and dicetyl phosphate at a mole ratio of 6:3:1 and total lipid concentration was 100 mg/mL. Solution of 0.05 M citrate buffer pH 5.5:PG (80:20) as an aqueous phase was used. Elastic liposomes composed of soybean phosphatidylcholine and Span® 80 at a weight ratio of 85:15 (Jain et al., 2003; Raza et al., 2011; Utreja, Jain, and Tiwary, 2011) was chosen based on skin permeation ability. An aqueous phase of elastic liposomes was a solution of 0.05 M citrate buffer pH 5.5:PG:ethanol (73:20:7). A niosome formulation was chosen based on skin permeation ability and modified from Waraporn Suwakul (2005). Niosomes were composed of Span® 20, cholesterol and Solulan® 75 at a weight ratio of 57.5:37.5:5 with total lipid content of 100 mg/mL. All the vesicular systems could be homogeneously formed without any phase separation

during storage at 4 ºC (in case of conventional liposomes and elastic liposomes) and at room temperature (in case of niosomes) for 2 days.

For microemulsions, the formulations composed of nonionic surfactant, ethanol or propylene glycol, and penetration enhancer oil such as oleic acid or isopropyl myristate were prepared. The formulations with the great skin penetration were chosen from literature reviews. Citrate buffer pH 5.5 was used to improve extract stability (นิริน อุดมสม และคณะ, 2548; Pornpen Kongaimpitee, 2007). The formulations of stable microemulsions selected from the preliminary study are shown in Table 1. The selected nanoemulsions are shown in Table 2.

Table 1 Microemulsions formulations selected from the preliminary study

Formulations	Composition		Modified from
	Ingredients	%w/w	
ME1	Oleic acid	5	Zhu et al., 2008
	Cremophor® EL	20	
	Ethanol	30	
	Citrate buffer pH 5.5	45	
ME2	Isopropyl myristate	5	Yuan et al., 2006
	Tween® 85	25	
	Ethanol	25	
	Citrate buffer pH 5.5	45	
ME3	Oleic acid	10	Park et al., 2005
	L.A.S.®	10	
	Ethanol	50	
	Citrate buffer pH 5.5	30	

Table 2 Nanoemulsions formulations selected from the preliminary study

Formulations	Composition		Modified from
	Ingredients	%w/w	
NE1	Caprylic/capric triglyceride	10	Wang et al., 2008
	Tween® 20	10	
	Citrate buffer pH 5.5	80	
NE2	Caprylic/capric triglyceride	10	Junyaprasert et al., 2009
	Tego® Care 450	10	
	Citrate buffer pH 5.5	80	
NE3	Soybean oil	8.5	Kakumanu et al., 2011
	Tween® 80	8.5	
	Citrate buffer pH 5.5	83	

3.1 Saturation solubility of the extract in nanosystem compositions

Estimated solubility of the selected extract in nanosystem compositions was determined in order to provide the maximum loading. The various solvents were used including 0.05 M citrate buffer pH 5.5, solution of 0.05 M citrate buffer pH 5.5:PG (80:20), solution of 0.05 M citrate buffer pH 5.5:PG:ethanol (73:20:7), caprylic/capric triglyceride, Cremophor® EL, isopropyl myristate, L.A.S.®, oleic acid, soybean oil, Span® 80, Tween® 20, Tween® 80 and Tween® 85. Solubility of the extract in Tego® Care 450 could not be determined because Tego® Care 450 is solid at room temperature. The solubility was examined as described in Part II (2.2) in triplicate.

3.2 The effect of preparation methods on entrapment efficiency of conventional liposomes

To obtain higher entrapment efficiency of liposomes, the method reported to give high entrapment efficiency such as microfluidization was studied and compared to film hydration method.

3.2.1 Saturation solubility of the extract in liposome compositions

Saturation solubility in liposome compositions of the selected extract from Part I was first determined to obtain the maximum extract loading. Saturated concentration of the extract in liposomes was determined from saturated concentrations of the extract in lipid phase and aqueous phase. The extract was dissolved in lipid phase at various concentrations. The concentration of extract in aqueous phase was kept constant at the saturated concentration of extract in solution of 0.05 M citrate buffer pH 5.5:PG (80:20) obtained from 3.1. Liposomes were then prepared using film hydration method. The maximum concentration of the extract in lipid phase, which any crystals were not observed under light microscope, was its saturation solubility in lipid phase. All experiments were performed in triplicate.

3.2.2 Preparation of liposomes

Liposomes containing soybean phosphatidylcholine, cholesterol and dicetyl phosphate at a mole ratio of 6:3:1 were prepared by film hydration and microfluidization techniques. Total lipid content of each formulation was 100 mg/mL.

Film hydration method was modified from the method of Pornpen Kongaimpitee (2007). Stock solutions of each component were prepared in chloroform. Then, appropriate volumes of stock solutions were pipetted into a 250 mL round bottom flask, mixed and eventually diluted with chloroform to 2 mL. Lipids were then deposited as a thin film in a round bottom flask by a rotator evaporator at 45 ºC (under pressure at 200 to 50 mbar, 80 rpm). The film was then hydrated with 1 mL of solution of 0.05 M citrate buffer pH 5.5:PG (80:20) containing the 90% saturated concentration

of extract at 40 ºC for 2 hours. All experiments were performed in triplicate. Liposomes were stored at 4 °C until used.

Microfluidization technique was modified from the method of Vemuri et al. (1990). Each lipid was accurately weighed into a beaker and mixed at 45 ºC. The solution of 0.05 M citrate buffer pH 5.5: PG (80:20) containing the extract at 90% saturated concentration was then added and mixed for 4 hours at 45 ºC. Then, the mixture was passed through a microfluidizer at 500 bar for 6 cycles at 40 ºC. All experiments were performed in triplicate. Liposomes were stored at 4 °C until used.

Liposomes prepared by both methods were characterized as mentioned in 3.4.1-3.4.3. Their entrapment efficiencies were then compared. The method providing better entrapment efficiency was also used for preparation of elastic liposomes.

3.3 Nanosystems for skin delivery of the selected *P. emblica* extract

In this study, conventional liposomes, elastic liposomes, niosomes, microemulsions and nanoemulsions were studied. Saturation solubility of the selected extract from Part I in each nanosystem was first determined to obtain the maximum extract loading. The nanosystems were prepared as the method described in 3.3.2. For the formulation containing the extract, the selected extract from Part I was incorporated at the 90% saturated concentration in each nanosystem from 3.2.1 and 3.3.1 to obtain the same thermodynamic activity of the extract across a membrane. Corresponding nanosystems without the extract were also prepared as blanks. All experiments were performed in triplicate. Then, all nanosystems were characterized and their stability was determined. Good appearance and stable formulations were then compared in *in vitro* release and *in vitro* skin permeation studies for suitable skin delivery of the extract.

3.3.1 Saturation solubility of the extract in nanosystems

Saturated concentrations of the extract in elastic liposomes and niosomes were determined as previously mentioned in 3.2.1. Solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) was used as an aqueous phase for elastic liposomes.

Solution of 0.05 M citrate buffer pH 5.5:PG (80:20) was used as an aqueous phase for niosomes.

Saturation solubility of the extract in microemulsions and nanoemulsions was evaluated by incremental addition of 0.10 g extract into 5 g of each formulation under magnetic stirring. Cloudiness or precipitate was visually observed when its concentration was above the saturated concentration. Once the approximate solubility was approached, the increment was refined to 0.01 g each time. If cloudiness or precipitate was visually observed, the experiment was repeated at step down extract concentration. The maximum extract concentration giving a transparent solution after adjusted pH to 5.5 with 5 %w/v sodium hydroxide was defined as the saturation solubility of extract in these microemulsions and nanoemulsions. All experiments were performed in triplicate.

3.3.2 Preparation of nanosystems

In this study, conventional and elastic liposomes were prepared by microfluidization. Both liposomes were stored at 4 ºC until used.

Niosomes were prepared using microfluidization technique. Each composition was accurately weighed into a beaker and mixed at 40-50 ºC. Solution of 0.05 M citrate buffer pH 5.5:PG (80:20) containing the extract at 90 % saturated concentration was then added and mixed for 6 hours at 45 ºC. Then, the formulation was passed through a microfluidizer at 1000 bar for 5 cycles at 45 ºC. Niosomes were stored at room temperature until used.

Microemulsions from Table 1 were prepared by mixing accurately weighed oil phase with surfactant and co-surfactant until homogenous by a magnetic stirrer. Then citrate buffer pH 5.5 was added and the mixture was stirred until clear solution was obtained. The 90 % of saturated concentration of the extract in each microemulsion obtained from 3.3.1 was added after these formulations were formed. The pH of each microemulsion was adjusted to 5.5 with 5 %w/v sodium hydroxide. Microemulsions were stored at room temperature until used.

Nanoemulsion formulations from Table 2 were prepared using microfluidization technique. Each composition of nanoemulsions was pre-mixed at 30 ºC using a high speed homogenizer at 6,000 rpm for 10 minutes and was passed through microfluidizer at 1,500 bar for 3 cycles. The 90 % of saturated concentration of the extract in each nanoemulsion obtained from 3.3.1 was added in pre-mixed process. Nanoemulsions were stored at room temperature until used.

3.4 Characterization of nanosystems

3.4.1 Morphology and visual determination

Conventional liposomes, elastic liposomes and niosomes were visually observed for homogeneous dispersion. Homogeneous formulations were then characterized by optical microscopy for the completeness of vesicle formation, morphology, aggregation and the absence of any extract crystals. Liposomes and niosomes were anisotropic and displayed birefringence under polarized microscopy (Bibi et al., 2011).

Microemulsions were visually observed for transparency, phase separation and precipitation after 7 days of storage at room temperature. The formation of microemulsions was verified by their isotropic nature using polarized light microscopy (Friberg, 1990).

Nanoemulsions were visually observed for phase separation and precipitation after 1 day of storage at room temperature. The formation of nanoemulsions was visually confirmed by bluish color. Nanoemulsions were prescreened by centrifugation at 3,500 rpm for 30 minutes. The formulations that did not show any phase separation were taken for further study.

3.4.2 Size and size distribution

Particle size and size distribution of liposomes, niosomes and nanoemulsions were measured using photon correlation spectroscopy (Zetasizer ZS) at room temperature (New, 1990). Samples were diluted with citrate buffer pH 5.5 for

intensity adjustment before measurement. Blank nanosystems were also determined. The measurement was done in triplicate.

3.4.3 Entrapment efficiency of the extract in liposomes and niosomes

To determine entrapment efficiency of the extract in liposomes and niosomes, the unentrapped extract was firstly separated from liposome and niosome dispersions by ultracentrifugation (New, 1990). The suspension was centrifuged at 65,000 rpm at 4 ºC for 20 hours. The supernatant was carefully separated from the pellet to determine the amount of unentrapped extract. Then the pellet was dissolved in methanol to analyze for the entrapped extract. The amount of extract was quantified by DPPH radical scavenging assay and tyrosinase inhibition assay as method mentioned in 3.4.5. Entrapment efficiency (EE) was calculated as follows:

$$\%EE = \frac{\text{Amount of extract in vesicles}}{\text{Amount of free extract+Amount of extract in vesicles}} \times 100\% \qquad (4)$$

3.4.4 Elasticity determination of elastic liposomes

To confirm the elasticity of elastic liposomes, their vesicles size and entrapment efficiency were determined before and after extrusion through polycarbonate membrane with 50 nm pore diameter (Jain et al., 2003) under pressure 500 bar. They were considered as elastic liposomes if they could pass under this condition without changes in their size and entrapment efficiency.

3.4.5 Content of the extract in nanosystems

The formulations were dissolved with methanol until clear solution was obtained. The solution was then diluted with methanol. The extract content was then analyzed by DPPH radical scavenging assay and tyrosinase inhibition assay as described in 1.1 and 1.2, respectively. For DPPH assay, methanol was used instead of absolute ethanol. For anti-tyrosinase assay, methanol was used instead of 60% PG. Both assays were partially validated as shown in Appendix C. Amount of extract was calculated from a calibration curve of percent DPPH inhibition or percent tyrosinase

inhibition versus the extract concentration in methanol. The extract content and percent labeled amount of extract in all nanosystems were calculated as follows:

$$\text{Extract content (mg/mL)} = \frac{\text{Amount of analysed extract (mg)}}{\text{Volume of nanosystem dispersion (mL)}} \quad (5)$$

$$\%\ \text{Labeled amount of extract} = \frac{\text{Actual extract content}}{\text{Theoretical extract content}} \times 100\% \quad (6)$$

3.4.6 Stability study of nanosystems

All nanosystems containing the extract were evaluated for physical and biological stabilities. All formulations were stored in glass containers wrapped with aluminum foil. Liposomes were kept at 4 °C while niosomes and microemulsions were kept at room temperature for 8 weeks. The sampling was performed every week. Changes in the properties of liposomes and niosomes were investigated for physical stability as mentioned in 3.4.1-3.4.3. Microemulsions were investigated as mentioned in 3.4.1.

Stability study of nanoemulsions was performed at accelerated condition and room temperature. At accelerated condition, nanoemulsions were taken for heating and cooling cycle for 6 cycles between 4 ºC and 45 ºC with storage at each temperature for 48 hr. The formulations that were stable at this condition were subjected to the freeze-thaw cycle test for 3 cycles between -21 ºC and +25 ºC with storage at each temperature for 48 hr (Pathan and Setty, 2011). The formulations that were stable at this condition were considered to be physically stable.

For biological stability, liposomes were kept at 4 ºC for 8 weeks. Niosomes, microemulsions and nanoemulsions were kept at room temperature for 8 weeks. All extract-loaded nanosystems were investigated for DPPH scavenging and anti-tyrosinase activities every 2 weeks. Percentage of the extract remaining in conventional liposomes, elastic liposomes and niosomes was sum of entrapped and free extract amounts obtained from 3.4.3. Percentage of the extract remaining in

microemulsions and nanoemulsions was determined for the extract content as described in 3.4.5.

Formulations which were physically and biologically stable were chosen for further *in vitro* release study and *in vitro* skin permeation study.

3.5 *In vitro* release study

Modified Franz diffusion cells were used to study *in vitro* release of the extract from nanosystems. In this experiment, a dialysis membrane (cellulose tubular membrane, Cellu-Sep®) with a molecular cut-off of 12,000-14,000 kDa was placed between the donor and the receptor compartment. The receptor compartment was filled with citrate buffer pH 5.5. The cells were maintained at a temperature of 37 ºC throughout the release study and were continuously stirred with a magnetic bar at 600 rpm. The receptor fluid and membrane were equilibrated to 37 ºC for 1 hour. One milliliter of an extract-loaded nanosystem or a control was placed on the donor compartment. The experiment was performed under occlusion. The 2.0 mL receptor fluid was withdrawn at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18 and 24 hours of study and was replaced with fresh receptor fluid equilibrated at 37 ºC. The samples were then analyzed for amount of the extract by DPPH radical scavenging assay as mentioned in 3.4.5. Triplicate of each experiment were performed. The percentage of the extract released from nanosystems and their controls were observed. Solution of citrate buffer pH 5.5:PG (80:20) containing the extract in 90% saturated concentration was used as a control for conventional liposomes and niosomes. Solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) containing the extract in 90% saturated concentration was used as a control for elastic liposomes. Solution of citrate buffer pH 5.5 containing the extract in 90% saturated concentration was used as a control for microemulsions and nanoemulsions. The cumulative amount of the extract released at each particular time was calculated (Yu and Liao, 1996). Percentage of the extract released at each time was calculated as equation (7).

$$\% \text{ Extract release} = \frac{\text{Cumulative amount of extract released at each time}}{\text{Amount of loading extract}} \times 100\% \quad (7)$$

3.6 *In vitro* skin permeation study

In vitro skin permeation of the extract in nanosystems was performed on modified Franz diffusion cell using full-thickness abdominal skin of new born pig (Schmook, Meingassner, and Billich, 2001) with non-occlusive condition. The receptor medium was phosphate buffer solution (PBS), pH 7.4. The skin was firstly set in place with the stratum corneum facing to the donor compartment and the dermal side facing to the receptor compartment. Residues were trimmed. The receptor fluid was then equilibrated to 37 ºC for an hour. After that, 200 µL/cm^2 of each formulation containing the extract or controls was placed on the skin surface of each cell.

At 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18 and 24 hours of study, the receptor fluid (2.0 mL) was collected and replaced with the equal volume of fresh receptor fluid equilibrated at 37 ºC. Samples were then analyzed for the extract amount by DPPH radical scavenging assay (Vicentini et al., 2009) as mentioned in 3.4.5. The cumulative amount of the extract permeating through the membrane after n^{th} sampling (Q_n) was calculated (Yu and Liao, 1996).

At the end of permeation study, the skin surface and donor compartment were rinsed 3 times with methanol which was then analyzed for the extract remaining on the donor compartment. The skin was then cut into small pieces and extracted with methanol (3 mL) by vortexing for 5 minutes, sonicating for 5 minutes, shaking at room temperature for 2 hours, and filtering through 0.45 µm membrane filter. The filtrate was then analyzed for the extract accumulated in the skin by DPPH radical scavenging assay. The controls used in this study were the same as in vitro release study. Six replicates of each experiment were performed.

The cumulative permeated amounts of the extract per diffusion area were plotted against times. The observed steady-state flux (J_{ss}) was obtained from the slope (Hofland et al., 1994). Q_s and Q_{24} were defined in this study as amount of the extract accumulated in skin and cumulative amount of the extract in the receiver per diffusion area at 24 hours, respectively. Percentage of the extract permeated at each time was calculated as equation (8) and then was plotted versus time.

$$\% \text{ Permeated extract} = \frac{\text{Cumulative amount of the permeated extract}}{\text{Amount of loading extract}} \times 100\% \quad (8)$$

Permeability coefficient (P_s) was calculated using equation (9)

$$P_s = J_{ss}/C_d \quad (9)$$

where C_d is the extract concentration in the donor compartment.

The enhancement factor (EF) of the formulation based on the permeability coefficient was defined as in equation (10).

$$EF = (P_s \text{ of the formulation})/(P_s \text{ of the control}) \quad (10)$$

The enhancement factor of the formulation based on the percentage of extract in the skin was defined as in equation (11).

$$EF \text{ of } \%Q_s = (\%Q_s \text{ of the formulation})/(\%Q_s \text{ of the control}) \quad (11)$$

The enhancement factor of the formulation based on the percentage of cumulative extract in the receptor medium at 24 hour was defined as in equation (12).

$$EF \text{ of } \%Q_{24} = (\%Q_{24} \text{ of the formulation})/(\%Q_{24} \text{ of the control}) \quad (12)$$

Relative flux (RF) was defined as in equation (13).

$$RF = (J_{ss} \text{ of the formulation})/(J_{ss} \text{ of the control}) \quad (13)$$

These parameters were compared with their controls, among each nanosystem and between nanosystems.

4. Statistical analysis

All data are shown as mean ± standard deviation (SD). One-way analysis of variance (One-way ANOVA) with a post-hoc comparison (Tukey or Dunnett 3T), was used to test the statistical significance at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

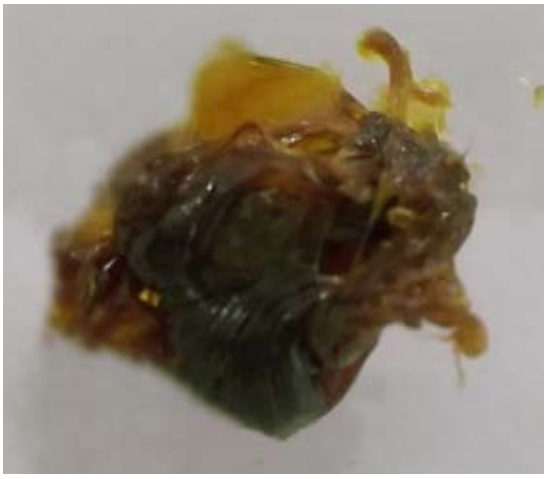
Part I Comparative evaluation of various *Phyllanthus emblica* fruit extracts using various extraction protocols

Extraction of *P. emblica* fruit has been practically done by using various methods. In this study, four extracts of *P. emblica* fruit: spray-dried powder of *P. emblica* compressed juice, spray-dried powder of ethanolic extract of fresh fruit, ethanolic extract of dried fruit and ethyl acetate extract of dried fruit, were investigated for the most active extract. The spray-dried powder of compressed juice and spray-dried powder of ethanolic extract of fresh fruit obtained from the study of Ubonthip Nimmannit et al (2009) were light yellow fine powders (Figures 2a and 2b). The ethanolic extract of dried fruit was reddish-brown semisolid (Figure 2c) with yield of 25.60 ± 2.72 %. The ethyl acetate extract of dried fruit was greenish-brown semisolid (Figure 2d) with yield of 1.32 %. The percent yield of the ethanolic extract of dried fruit of this study was close to that of Panithi Raknam et al. (2009) (23.40 %). But, it was higher than that of Boontarika Kawswang et al. (2009) (9.84%), who prepared the ethanolic extract of fresh *P. emblica* fruit using percolation method. This was due to the different sources, characteristic of plant raw material, and extraction methods.

(a) Spray-dried powder of compressed juice

(b) Spray-dried powder of ethanolic extract of fresh fruit

(c) Ethanolic extract of dried fruit



(d) Ethyl acetate extract of dried fruit

Figure 2 Physical appearance of the *P. emblica* fruit extracts prepared by various methods

Biological activities and total phenolic content of the four *P. emblica* crude extracts were determined and compared.

1.1 DPPH radical scavenging activity

The antioxidant activity of *P. emblica* crude extracts was determined spectrophotometically by DPPH radical scavenging assay. From the investigation, it was found that as the extract concentration increased, the extent of DPPH inhibition increased. All *P. emblica* crude extracts and controls posed various degrees of free

radical scavenging activity (Table 3). Ethyl acetate extract of dried fruit showed significant higher than ethanolic extract of dried fruit in regard to free radical scavenging activity ($p < 0.05$). Both dried fruit extracts exhibited significantly higher scavenging effect than the two spray-dried powders ($p < 0.05$). Comparing to the control standards, all extracts had significantly lower antioxidant activity than that of ascorbic acid ($p < 0.05$). However, the ethanolic extract and the ethyl acetate extract of dried fruit had significantly higher antioxidant activity than that of gallic acid ($p < 0.05$). The result was in accordance to Panithi Raknam et al. (2009), who reported that their ethanolic extract gave slightly lower antioxidant activity (IC_{50} = 2.48 ± 0.004 µg/mL) than that of ascorbic acid (IC_{50} = 2.18 ± 0.007 µg/mL). Sirirat Pinsuwan et al. (2007) also prepared the ethanolic extract of dried *P. emblica* fruit using maceration. This extract posed closely antioxidant activity (IC_{50} = 1.55 ± 0.35 µg/mL) to that of this study. For the ethyl acetate extract of dried *P. emblica* fruit, antioxidant activity of the extract in this study was in contrast with that prepared by soxhlet extraction of Jantima Homklob et al. (2010). The ethyl acetate extract showed higher antioxidant activity (IC_{50} = 0.025 - 0.037 mg/mL) than that of ascorbic acid (IC_{50} = 0.089 ± 0.002 mg/mL).

Table 3 DPPH radical scavenging activity of various *P. emblica* crude extracts and reference compounds (n = 3, mean ± SD)

Extracts and reference compounds	IC_{50} (µg/mL)
Spray-dried powder of compressed juice	5.546 ± 0.044
Spray-dried powder of ethanolic extract of fresh fruit	4.707 ± 0.029
Ethanolic extract of dried fruit	1.221 ± 0.005
Ethyl acetate extract of dried fruit	1.172 ± 0.032
Ascorbic acid	0.953 ± 0.004
Gallic acid	1.426 ± 0.003

1.2 Tyrosinase inhibition activity

All crude extracts were analyzed for their tyrosinase inhibition activity**.** Their IC_{50} values are presented in Table 4. The significant differences among all crude extracts were observed ($p < 0.05$). The ethyl acetate extract of dried fruit showed the highest anti-tyrosinase activity followed by the ethanolic extract of dried fruit, spray-dried powder of ethanolic extract of fresh fruit and spray-dried powder of *P. emblica* compressed juice**,** respectively. All extracts had significantly lower tyrosinase inhibition activity than that of kojic acid ($p < 0.05$). However, the ethanolic extract of dried fruit and the ethyl acetate extract of dried fruit were significantly higher tyrosinase inhibition activity than that of gallic acid ($p < 0.05$). The result was in the range shown in the study of Jantima Homklob et al. (2010) who prepared the ethyl acetate extract of dried *P. emblica* fruit from different regions in Thailand using soxhlet extraction. Tyrosinase inhibition activity of these extracts was at IC_{50} of 0.151- 0.710 mg/mL and was lower than that of kojic acid (IC_{50} = 0.050 ± 0.007 mg/mL). A similar result was also found in the study of Boontarika Kawswang et al. (2009). They reported that the ethyl acetate extract prepared using partition method showed nearly anti-tyrosinase activity to that of gallic acid but it was lower (IC_{50} of more than 1 mg/mL) than kojic acid (0.0902 mg/mL).

Table 4 Anti-tyrosinase activity of various *P. emblica* crude extracts and reference compounds (n = 3, mean ± SD)

Extracts and reference compounds	IC_{50} (mg/mL)
Spray-dried powder of compressed juice	1.868 ± 0.026
Spray-dried powder of ethanolic extract of fresh fruit	0.875 ± 0.013
Ethanolic extract of dried fruit	0.519 ± 0.007
Ethyl acetate extract of dried fruit	0.425 ± 0.006
Kojic acid	0.027 ±0.0001
Gallic acid	0.572 ± 0.003

1.3 Total phenolic content

Phenolic compounds are active compounds which are responsible to antioxidant and tyrosinase inhibition activity of *P. emblica* extract (Majeed et al., 2008; Zhang et al., 2001). Total phenolic content of *P. emblica* extract was determined using Folin-Ciocalteau method. The total phenolic content was significantly different among all extracts ($p < 0.05$). From Table 5, ethyl acetate extract of dried fruit exhibited the highest total phenolic content followed by ethanolic extract of dried fruit, spray-dried powder of ethanolic extract of fresh fruit and spray-dried powder of compressed juice, respectively. Total phenolic content of the ethanolic extract of this study was more than that reported by Sirirat Pinsuwan et al. (2007) (454.7 ± 27.0 mg GAE/g). However, total phenolic content of the ethyl acetate extract of this study was in a range of Jantima Homklob et al. (2010) (345 - 597 mg GAE/g) which prepared by soxhlet extraction.

Table 5 Total phenolic content of various *P. emblica* crude extracts (n = 3, mean ± SD)

Extracts	Total phenolic content (mg GAE/g)
Spray-dried powder of compressed juice	160.656 ± 0.944
Spray-dried powder of ethanolic extract of fresh fruit	235.831 ± 2.274
Ethanolic extract of dried fruit	490.756 ± 0.185
Ethyl acetate extract of dried fruit	527.535 ± 1.637

The results in 1.1 - 1.3 showed that higher biological activities of *P. emblica* extracts was consistent with their higher total phenolic content. In a correlation study (Figures 3 and 4), total phenolic content were linearly correlated to the antioxidant activity (r = 0.999) and tyrosinase inhibition activity (r = 0.999) of the ethanolic *P.emblica* extract of dried fruit. This could be revealed that the antioxidant activity and tyrosinase inhibition activity of the *P. emblica* extract was attributed to its phenolic compounds.

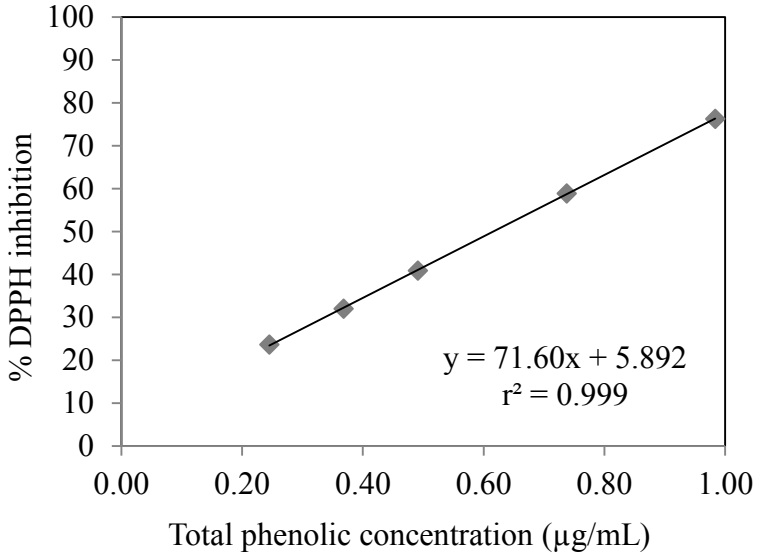


Figure 3 Correlation between total phenolic content and antioxidant activity of the ethanolic extract of dried fruit

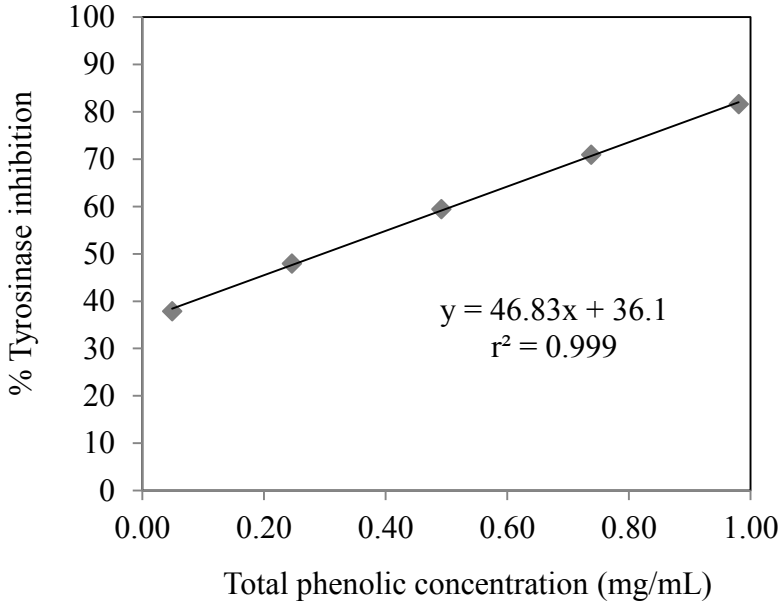


Figure 4 Correlation between total phenolic content and tyrosinase inhibition activity of the ethanolic extract of dried fruit

From all data above, the ethanolic and ethyl acetate extracts gave higher antioxidant activity, tyrosinase inhibition activity and total phenolic content than the spray-dried powder of ethanolic extract of fresh fruit and spray-dried powder of

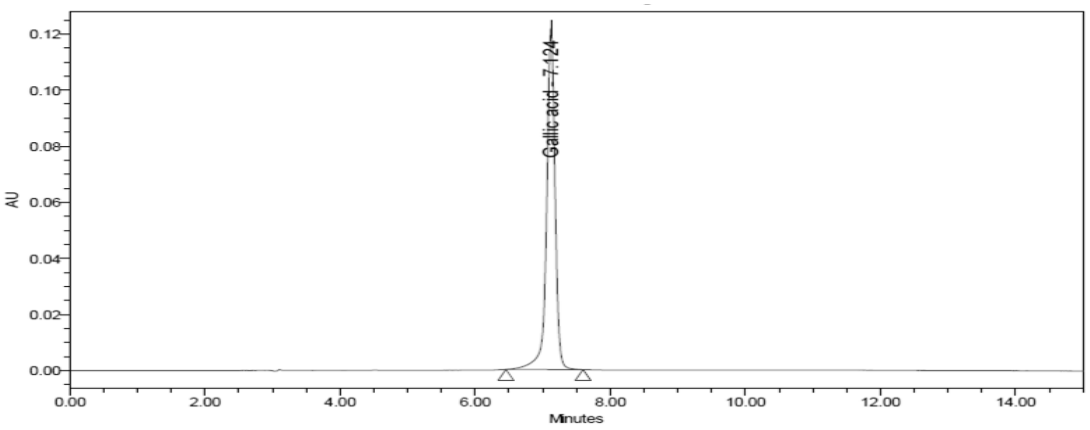
compressed juice. The result was attributed to the polarity properties of extraction solvents and active compounds. Since the expected active compounds in *P. emblica* extract are semi-polar compounds, such as tannins, benzenoid, and flavonoids, the extraction using solvents such as ethanol and ethyl acetate gives the higher active extract.

Although the ethanolic extract of dried fruit posed slightly lower biological activities and total phenolic content than the ethyl acetate extract of dried fruit, it gave considerably higher yield content. The ethanolic extract of dried fruit was thus more economical to be produced and used as an active composition for the further study.

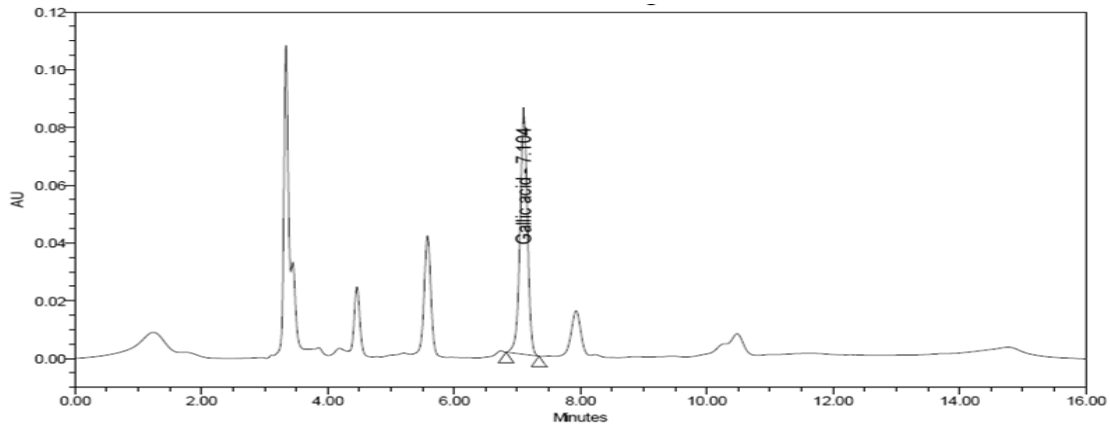
Part II Evaluation of physicochemical properties of the selected crude extract

2.1 Quantification of gallic acid content

The HPLC system was used to quantify gallic acid content in the ethanolic extract of dried fruit. Chromatogram of the extract (Figure 5a) had one of major peaks matched to peak of standard gallic acid (Figure 5b), which was at the retention time about 7.1 minutes. It was found that 1 g of the ethanolic extract of dried fruit had 23.894 ± 0.312 mg of gallic acid. The gallic acid content in this study was in a range reported by Poltanov et al. (2009) who quantified gallic acid content of the different water-soluble *P. emblica* fruit extracts ranging from 23.22 to 105.4 mg/g. However, it was less than the ethanolic extract prepared by maceration of Sirirat Pinsuwan et al. (2007) which was 34.14 mg/g. The result was attributed to different sources of raw material.



(a)



(b)

Figure 5 HPLC Chromatograms of standard gallic acid (a) and the ethanolic extract of dried fruit (b)

2.2 Saturation solubility of the extract in water and ethanol

In this study, the solubility of the ethanolic extract of dried fruit could not be exactly determined by extract assay. When the concentration of the extract was increased, the soluble active constituent was continuously dissolved but some insoluble compounds were precipitated. The estimated solubility of the ethanolic extract was practically done by observation of a clear solution. The extract was dissolved in water and ethanol at room temperature. The maximum extract concentration giving a transparent solution was defined as saturation solubility of the extract in that particular solvent. The saturation solubility of the ethanolic extract is shown in Table 6. As a

result, the crude extract was difficult to be dissolved in either water or ethanol because insoluble compounds were precipitated. This could be a problem for further formulation. Therefore, a re-dissolution process was performed to remove insoluble compounds and to obtain higher soluble extract.

Table 6 Saturation solubility of the extract in water and ethanol (n = 3, mean ± SD)

Solvents	Solubility (mg/mL)
Water	0.378 ± 0.006
Ethanol	0.800 ± 0.020

2.3 Re-dissolution process of the crude extract

In this study, the optimal re-dissolution process for obtaining higher soluble extract with high activity was investigated based on the highest anti-tyrosinase activity as follows.

2.3.1 The effect of extract concentrations on anti-tyrosinase activity

To find the suitable concentration of the extract, the extract in water and absolute ethanol were prepared at the concentrations of 10, 15, 20 and 25 g/100 mL. The mixtures were sonicated for 45 minutes and then filtered through Whatman no. 5 filter paper under vacuum. The solution from each concentration was diluted with methanol to 100-fold before examining for tyrosinase inhibition activity as the method described in Chapter III, 1.2. All experiments were performed in triplicate. Results showed that percent tyrosinase inhibition of the extract solution increased when the concentration of the extract in both water and ethanol increased (Figure 6). The ethanolic solution exhibited higher percent tyrosinase inhibition than the water solution. It could be indicated that the active was dissolved in ethanol better than in water. At the concentrations of 20 and 25 g per 100 mL of ethanol, their percent tyrosinase inhibition was insignificantly different because its saturated concentration was reached. Thus, the extract concentration of 20 g per 100 mL of ethanol was chosen for the further study.

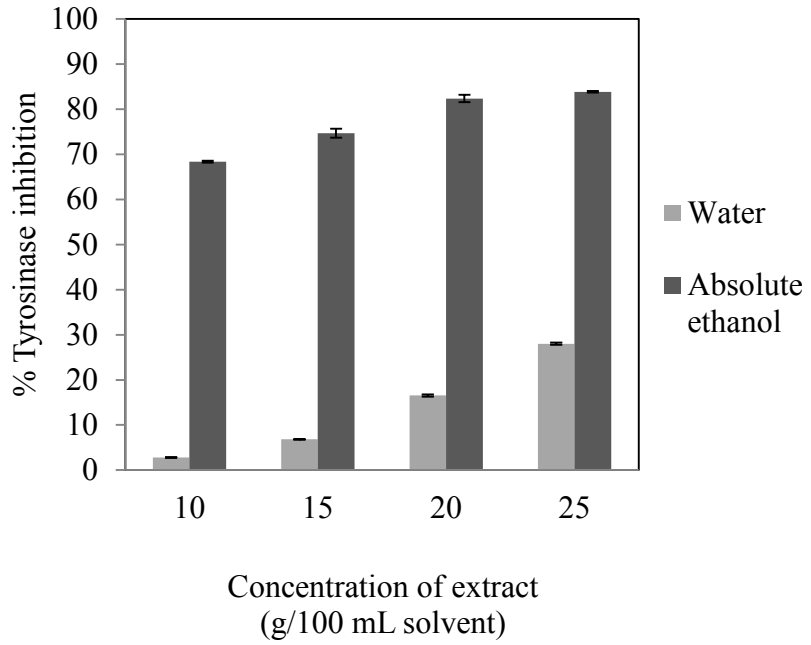


Figure 6 The effect of extract concentrations in water and ethanol on tyrosinase inhibition activity (n = 3)

2.3.2 The effect of sonication time on anti-tyrosinase activity

To ascertain optimal sonication time, the optimal concentration of extract (20 g/100 mL of absolute ethanol) was prepared with various sonication time durations (30, 45, 60, 90 and 120 minutes). The mixture was then filtered through Whatman no.5 filter paper under vacuum. The solution from each concentration was diluted with methanol to 100-fold before examining for tyrosinase inhibition as the method described in Chapter III, 1.2. All experiments were performed in triplicate. It was found that sonication time was directly proportional to percent tyrosinase inhibition (Figure 7). The longer sonication time, the extract posed higher percent tyrosinase inhibition. It was indicated that dissolution of the extract was increased by sonication time. However, the longer sonication time increased temperature of the solution up to 38 ºC which might affect the stability of the extract. Thus, the optimal sonication duration of 60 minutes was selected for the further study.

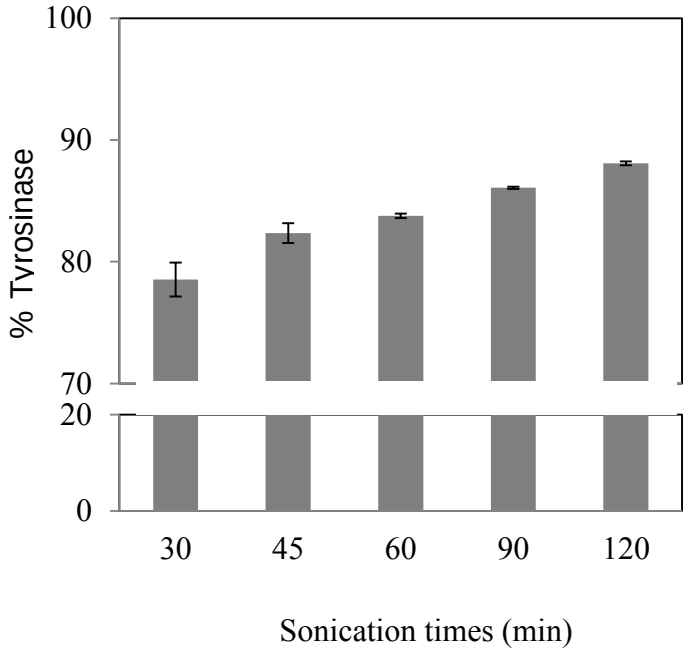


Figure 7 The effect of sonication time on tyrosinase inhibition activity (n = 3)

2.3.3 The effect of percentage of ethanol in purified water on anti-tyrosinase activity

The optimal concentration of extract (20 g/100 mL of ethanol) was prepared in various percentages of ethanol in water (50 %v/v, 65 %v/v, 85 %v/v, 95 %v/v and 99.9% v/v) and was sonicated for 60 minutes. The mixture was then filtered through Whatman no. 5 filter paper under vacuum. The solution from each concentration was diluted with methanol to 100-fold before examining for tyrosinase inhibition activity as the method described in Chapter III, 1.2. All experiments were performed in triplicate. The result showed that the higher percentage of ethanol up to 95%, the higher percentage of tyrosinase inhibition (Figure 8). At 95 %v/v and 99.9 %v/v of ethanol, there were insignificant differences in percent tyrosinase inhibition because the saturation solubility of the extract was reached. As a result, 95 %v/v ethanol was an optimal percentage of ethanol for re-dissolution process of the extract.

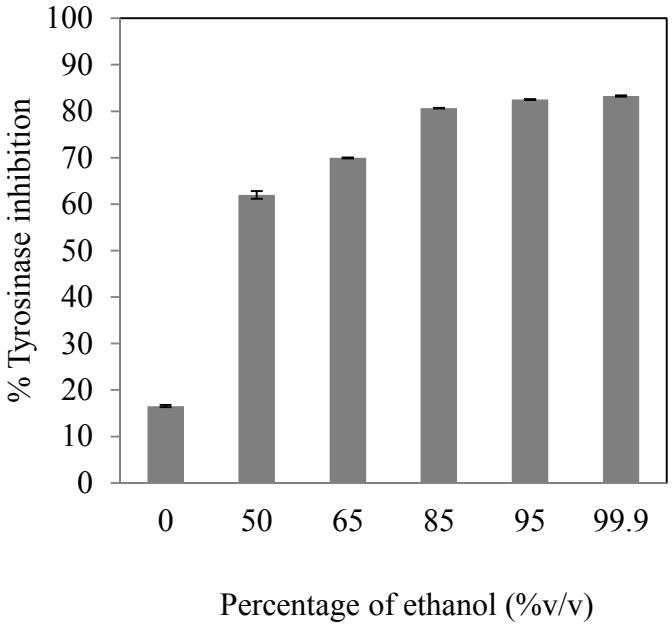


Figure 8 The effect of percentage of ethanol in purified water on tyrosinase inhibition activity (n = 3)

2.4 Evaluation of the dissolvable crude extract

Regard to the optimal re-dissolution process obtained from 2.3, the "dissolvable extract" was prepared by dissolving 20% of the ethanolic extract from dried *P. emblica* fruit in 95 %w/v ethanol with 60-minute sonication. The mixture was then filtered through Whatman no. 5 filter paper under vacuum. The filtrate was dried at 40 °C using a rotary evaporator and left on water bath (45 ºC) until the constant weight was reached. An extract in tightly glass containers were kept in a refrigerator until used. The obtained extract was reddish-brown sticky semisolid being glossier and slightly lower viscous than the initial extract (Figure 9). Percent yields of the dissolvable extract were similar among batches (Table 7), which indicated that the preparation method was reproducible.

Figure 9 Physical appearance of the dissolvable extract

Table 7 Yields of the dissolvable extract

Batches	Weight of the initial extract (g)	Weight of the obtained extract (g)	%Yield
1	30	27.52	91.73
2	15	13.50	90.00
3-1	15	13.57	90.49
3-2	200	178.86	89.54

The dissolvable extract was determined for DPPH inhibition activity, tyrosinase inhibition activity and total phenolic content as the methods previously mentioned in Chapter III, 1.1-1.3. As shown in Table 8, the dissolvable extract exhibited significantly higher capacity to scavenge free radicals against $DPPH^{\bullet}$ and contained higher total phenolic content compared to the initial extract. Its tyrosinase inhibition activity did not drastically change from that of the initial extract.

Table 8 Comparison between the initial crude extract and the dissolvable crude extract (n = 3, mean ± SD)

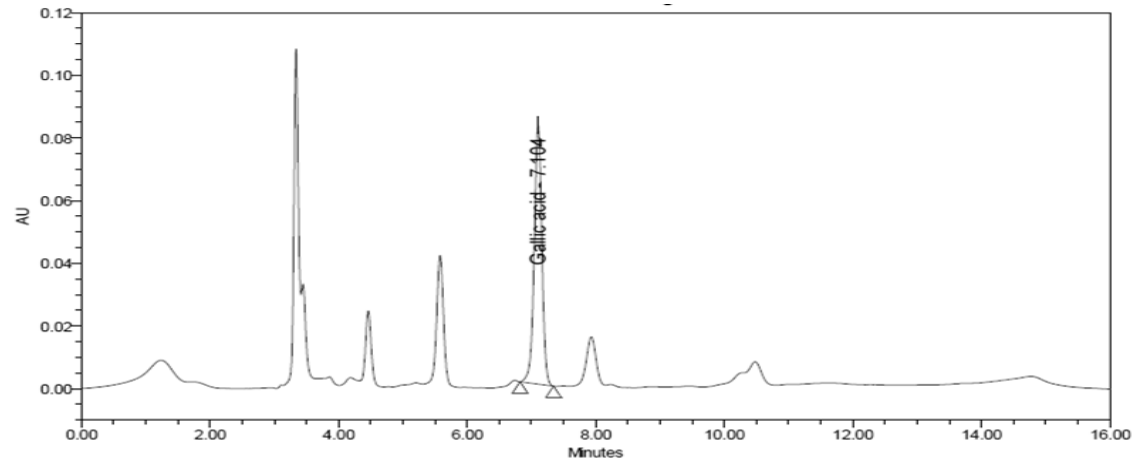
	Initial extract	Dissolvable extract
DPPH inhibition (IC_{50}, µg/mL)	1.221 ± 0.005	0.878 ± 0.006
Tyrosinase inhibition (IC_{50}, mg/mL)	0.519 ± 0.007	0.539 ± 0.005
Total phenolic content (mg GAE/g)	490.756 ± 0.185	504.688 ± 0.419
Gallic acid content (mg/g)	23.894 ± 0.312	26.630 ± 0.198
Solubility (mg/mL)		
• Water	0.378 ± 0.006	0.600 ± 0.021
• Ethanol	0.800 ± 0.020	5.003 ± 0.175
• Propylene glycol	ND	143.495 ± 0.691

ND = not determined

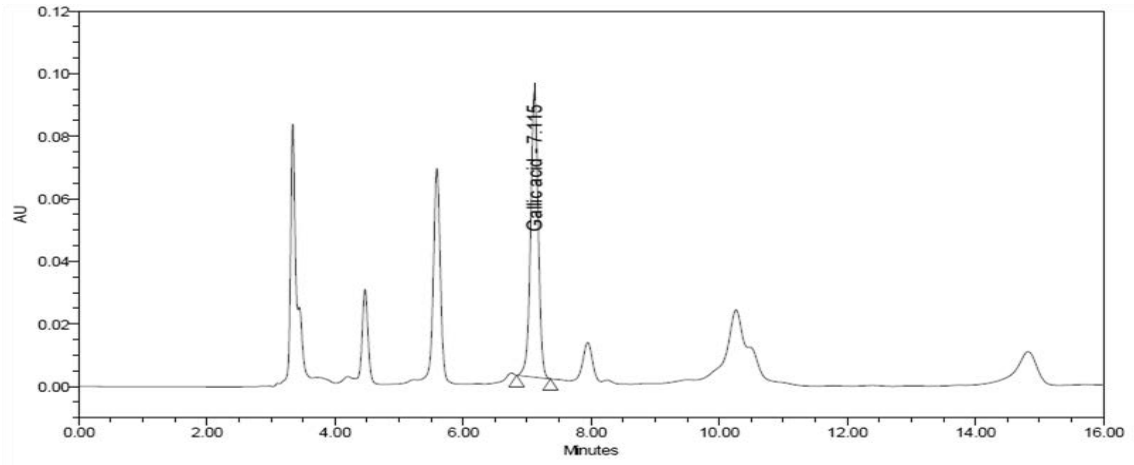
From HPLC experiment (Figure 10), chromatograms of both crude extracts were similar but difference in concentration of gallic acid. The concentration of gallic acid was significantly higher ($p < 0.05$) in the dissolvable extract than the initial extract (Table 8).

Moreover, the solubility of the dissolvable extract in water and absolute ethanol at room temperature were significantly better ($p < 0.05$). Results indicated that some insoluble and inactive compounds were removed from the crude extract during the re-dissolution process. As a result, the dissolvable extract was used for further preparation of nanosystems.

In addition, the saturation solubility of the extract in propylene glycol was determined. The result showed that the saturation solubility of the dissolvable extract was the highest in propylene glycol (Table 8). So in this study, propylene glycol and ethanol were preferred in the nanosystem formulations to improve drug loading.



(a)



(b)

Figure 10 HPLC chromatograms of the ethanolic extract of dried *P. emblica* fruit (a) and the dissolvable extract (b)

Part III Comparison of various nanosystems for skin delivery of the selected *P. emblica* fruit extract

3.1 Saturation solubility of the extract in nanosystem compositions

In this experiment, the solubility of the dissolvable extract from 2.4 was determined in nanosystem compositions for the maximum loading of extract in each nanosystem. The data (Table 9) showed that the dissolvable extract dissolved better in solvent mixtures containing propylene glycol than oils and surfactants because of the highest solubility in propylene glycol.

Table 9 Saturation solubility of the extract in nanosystem compositions (n = 3, mean ± SD)

Solvents	Temperature (ºC)	Solubility (mg/g)
0.05 M citrate buffer pH 5.5	RT	0.837 ± 0.017*
Solution of 0.05 M citrate buffer pH 5.5:PG (80:20)	RT	5.673 ± 0.021*
	4 ºC	5.678 ± 0.010*
Solution of 0.05 M citrate buffer pH 5.5:PG: ethanol (73:20:7)	4 ºC	6.204 ± 0.005*
Caprylic/capric triglyceride	RT	0.038 ± 0.061
Cremophor® EL	RT	0.099 ± 0.017
Isopropyl myristate	RT	0.041 ± 0.023
L.A.S.®	RT	0.118 ± 0.018
Oleic acid	RT	0.049 ± 0.014
Soybean oil	RT	0.081 ± 0.013
Span® 80	4 ºC	0.023 ± 0.011
Tween® 20	RT	0.172 ± 0.018
Tween® 80	RT	0.151 ± 0.024

* solubility in mg/mL

3.2 The effect of preparation methods on entrapment efficiency of conventional liposomes

Two preparation methods were studied to improve entrapment efficiency of conventional liposomes. The saturation solubility study showed that the extract at minimum studied concentration (0.01 mg/100 mg lipid) could not dissolve in lipid compositions of conventional liposomes. At this concentration, precipitate was visually observed. Therefore, the estimated solubility of the extract in conventional liposomes was defined as the saturation solubility of the extract in solution of 0.05 M citrate buffer pH 5.5:PG (80:20) at 4 ºC (5.678 ± 0.010 mg/mL).

After that, liposomes containing the extract at 90% saturated concentration (5.11 mg/mL) was prepared by both film hydration and microfluidization techniques. A maltese cross observed under polarized microscopy confirmed the formation of liposomes prepared by film hydration (Figure 11). However, vesicle size of liposomes prepared by microfluidization was too small to be directly observed under the polarized microscopy.

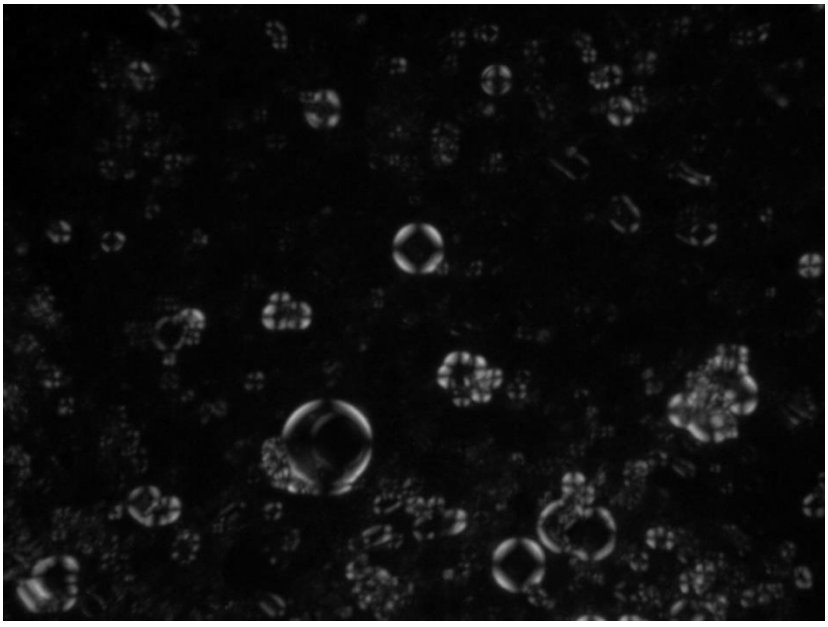


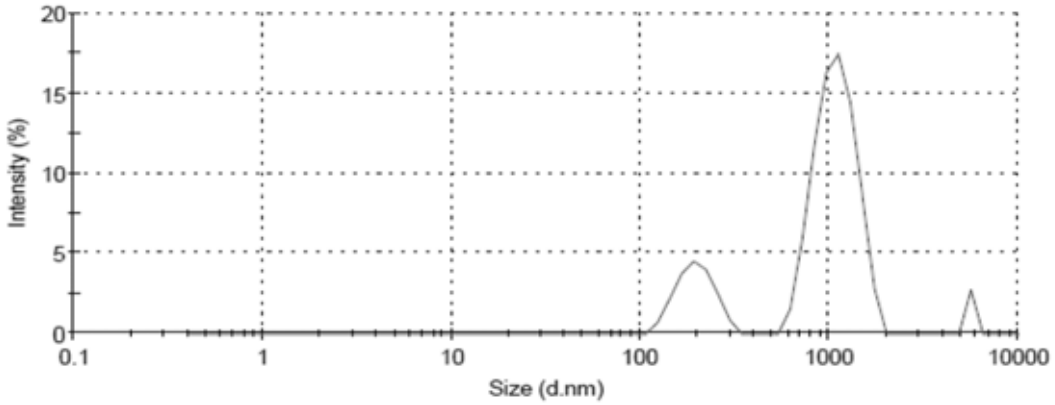
Figure 11 Appearance of conventional liposomes prepared by film hydration observed under polarized microscopy

The liposomes prepared by microfluidization were more translucent liquid than that prepared by film hydration (Figure 12). This was because the particle size of liposomes prepared by microfluidization was smaller than that prepared by flim hydration.

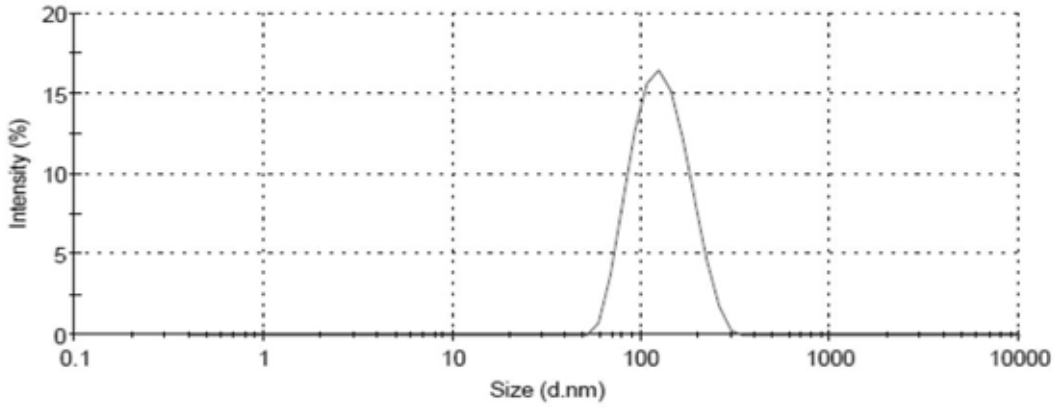
(a) (b)

Figure 12 Physical appearance of the extract-loaded liposomes prepared using film hydration (a) and microfluidization (b)

The liposomes prepared by film hydration formed heterogeneous vesicles (PDI = 0.763 - 1.000) with vesicle size of 1,286.78 ± 68.70 nm (Figure 13a). In contrast, the liposomes prepared by microfluidization formed uniform vesicles (PDI = 0.110 - 0.152) with vesicle size of 118.83 ± 0.95 nm (Figure 13b). By microfluidization, multiple collisions of two dispersion streams in the interaction chamber leaded to collapse and reformation of the vesicle membrane. This resulted in decreases in both size and size distribution of liposomes (Jahn et al., 2004).



(a)



(b)

Figure 13 Size distribution of the extract-loaded liposomes prepared using film hydration (a) and microfluidization (b)

The entrapment efficiency was determined by ultracentrifugation method. The entrapped and free extract was quantified only by DPPH radical scavenging activity. Tyrosinase inhibition activity could not be determined because clear solution could not be obtained when the formulation was diluted to the desired concentration. From Table 10, the result indicated that preparation methods of liposomes affected the entrapment efficiency of the extract. There was considerable improvement in entrapment efficiency when microfluidization was used for liposomal preparation compared to film hydration method. The result was because larger liposomes can be cut down and rearranged in the interaction chamber during microfluidization process (Cong et al., 2010). Microfluidization was also reported to improve the entrapment

efficiency of liposomes containing hemoglobin (Zheng et al., 1999) and more than 93% entrapment efficiency of pirarubicin liposomes was obtained by microfluidization (Cong et al., 2010). Microfluidization is a continuous process for liposome preparation and is applicable for large-scale production (Pegg and Shahidi, 2007). Consequently, microfluidization was selected for further preparation of elastic liposomes.

Table 10 Entrapment efficiency of the extract-loaded liposomes prepared using film hydration and microfluidization (n = 3, mean ± SD)

Preparation methods	% Entrapment efficiency
Film hydration	42.72 ± 0.23
Microfluidization	53.67 ± 0.31

3.3 Alternative delivery systems

3.3.1 Saturation solubility of the extract in nanosystems

Similar to liposomes, the 0.01 mg of extract per 100 mg lipid (minimum studied concentration) could not be dissolved in lipid compositions of elastic liposomes and niosomes. Therefore, the estimated solubility of the extract in elastic liposomes and niosomes were defined as the saturation solubility of the extract in solution of 0.05 M citrate buffer pH 5.5:PG:ethanol (73:20:7) (6.204 ± 0.005 mg/mL) and solution of 0.05 M citrate buffer pH 5.5:PG (80:20) at room temperature (5.673 ± 0.021 mg/mL), respectively. Based on the saturation solubility of the extract, the 90% saturated concentrations of the extract loaded into elastic liposomes and niosomes were 5.58 and 5.11 %w/w, respectively.

The saturation solubility of the extract in microemulsions after pH adjustment to 5.5 using 5 %w/v NaOH is shown in Table 11. The result showed that the saturation solubility of the extract in formulation ME1 was the highest followed by the formulation ME3 and ME2, respectively. High solubility of extract in microemulsions was due to high content of surfactant and ethanol content which was good solvents for the extract.

Table 11 Saturation solubility of the extract in microemulsions and their pH before and after adjusting pH with 5 %w/v NaOH (n = 3, mean ± SD)

Formulation	Saturation solubility (g/100 g)	pH value before pH adjustment	pH value after pH adjustment
ME1	6.758 - 6.762	4.74 ± 0.03	5.52 ± 0.04
ME2	3.627 - 3.644	5.03 ± 0.03	5.50 ± 0.02
ME3	4.515 - 4.531	4.95 ± 0.04	5.51 ± 0.01

Based on the saturation solubility of the extract, the 90% saturated concentrations of the extract loaded into the formulation ME1, ME2 and ME3 were 6.08, 3.28 and 4.08 %w/w, respectively.

Saturation solubility of the extract in formulation is shown in Table 12. The saturation solubility of the extract in formulation NE3 was the highest followed by the formulation NE1 and NE2, respectively. The higher saturation solubility of the extract in the formulations NE1 and NE3 were because of higher solubility of the extract in their compositions.

Table 12 Saturation solubility of the extract in nanoemulsions and their pH values (n = 3, mean ± SD)

Formulation	Saturation solubility (g/100 g)	pH value
NE1	2.345	5.50 ± 0.01
NE2	1.607 - 1.608	5.51 ± 0.01
NE3	2.579	5.51 ± 0.02

Based on the saturation solubilities of the extract, the 90% saturated concentrations of extract loaded into the formulation NE1, NE2 and NE3 were 2.11, 1.45 and 2.32 %w/w, respectively.

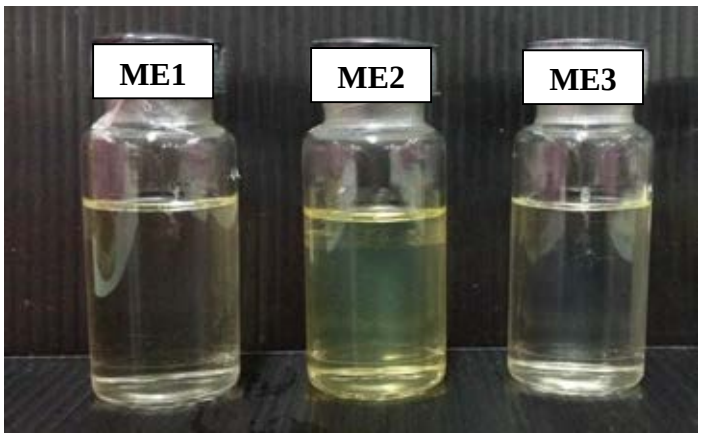
3.3.2 Morphology and visual appearance of nanosystems

The extract-loaded elastic liposomes were translucent liquid as shown in Figure 14a. The obtained niosomes were homogeneous opalescent dispersion as shown in Figure 14b.

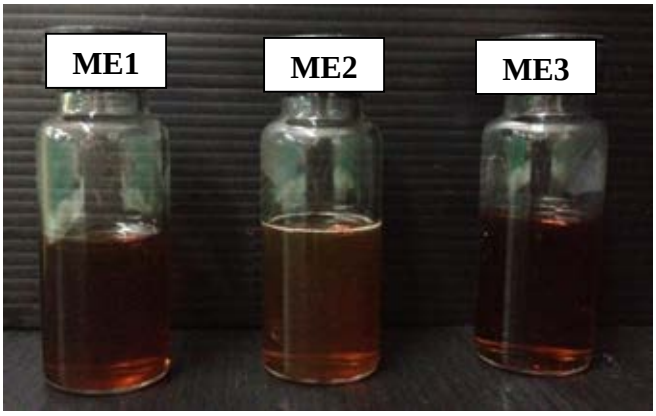
(a) (b)

Figure 14 Physical appearance of the extract-loaded elastic liposomes (a) and niosomes (b) prepared using microfluidization

For microemulsions, the formulations ME1, ME2 and ME3 were light yellow, optically transparent, and low viscous liquids without phase separation (Figure 15a). The dilution test was used to identify types of the microemulsions. Microemulsions were diluted with water or oil used in the formulations. The microemulsions which could be diluted with water were defined as o/w microemulsions. On the other hand, microemulsions which could be diluted with oil were defined as w/o microemulsion. The result showed that all formulations were o/w microemulsions. After the addition of the extract, all formulations were reddish-brown, transparent, and low viscous liquid as shown in Figure 15b. Their color intensity varied depending on the concentration of the extract loaded.



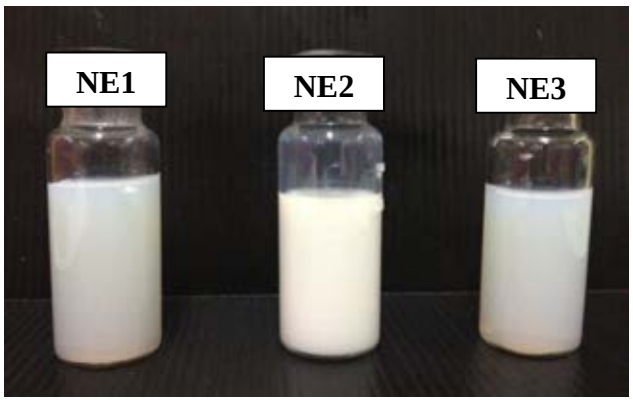
(a)



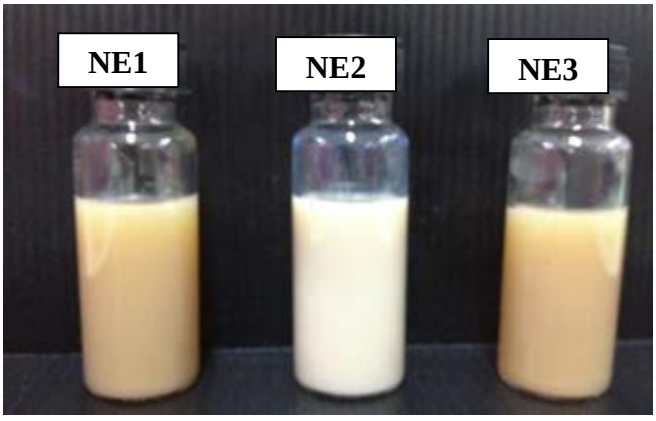
(b)

Figure 15 Physical appearance of the blank microemulsions (a) and the extract-loaded microemulsions (b)

Appearance of nanoemulsions is shown in Figure 16a. The formulations NE1 and NE3 were translucent liquids while the formulation NE2 was more opaque because vesicle size of NE2 was larger than that of NE1 and NE3 (Table 13). Physical appearance of all extract-loaded nanoemulsions is shown in Figure 16b. All formulations were light brown and translucent liquids. Their color intensity varied depending on the concentration of extract added.



(a)



(b)

Figure 16 Physical appearance of the blank nanoemulsions (a) and the extract-loaded nanoemulsions (b)

3.3.3 Size and size distribution

Particle size and size distribution of conventional liposomes, elastic liposomes, niosomes and nanoemulsions were measured using photon correlation spectroscopy. The data are shown in Table 13. Droplet sizes of all formulations were in the range of nanometers. Their size distributions were narrow (Table 13, Figures 17 and 18). When the extract was incorporated, particle sizes of all formulations were comparable to that of blank formulations. It showed that the extract did not affect particle sizes of the formulations.

Table 13 Size and size distribution of liposomes, niosomes and nanoemulsions (n = 3, mean ± SD)

Nanosystems	Size (nm)	Polydispersity index
Blank conventional liposomes	120 ± 0.76	0.113 - 0.163
Blank elastic liposomes	151 ± 1.463	0.098 - 0.146
Blank niosomes	644.61 ± 1.39	0.173 - 0.252
The extract-loaded conventional	118.71 ± 0.74	0.110 - 0.152
liposomes	152.46 ± 0.66	0.120 - 0.163
The extract-loaded elastic liposomes	643.87 ± 1.76	0.192 - 0.261
The extract-loaded niosomes	63.40 ± 2.41	0.118 - 0.140
Blank NE1	150.97 ± 4.58	0.082 - 0.136
Blank NE2	57.83 ± 2.73	0.180 - 0.191
Blank NE3	58.95 ± 0.407	0.106 - 0.152
The extract-loaded NE1	162.08 ± 1.313	0.124 - 0.192
The extract-loaded NE2	75.15 ± 0.792	0.199 - 0.219
The extract-loaded NE3		

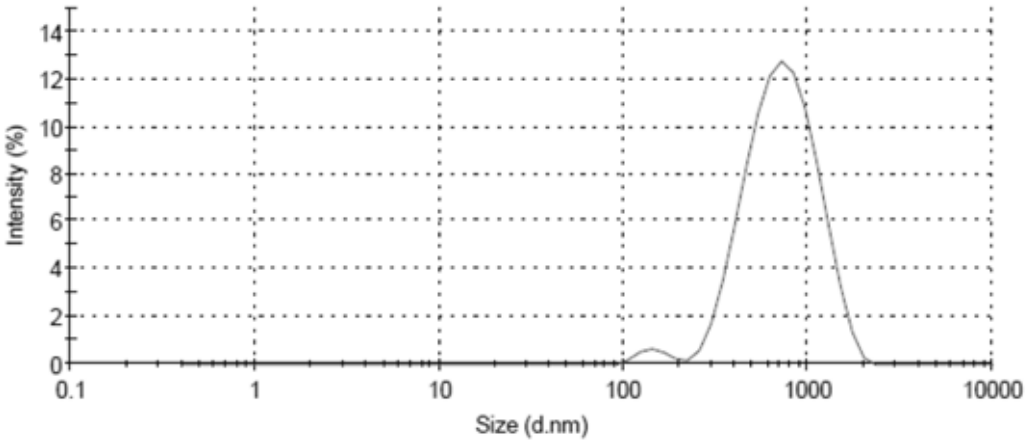
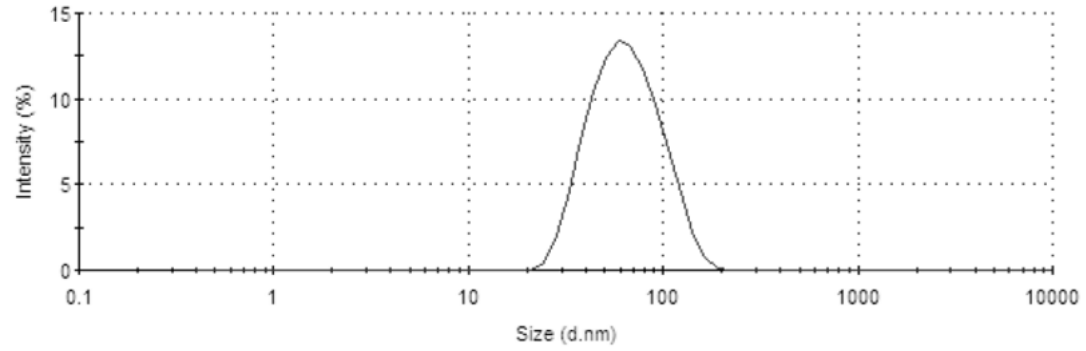
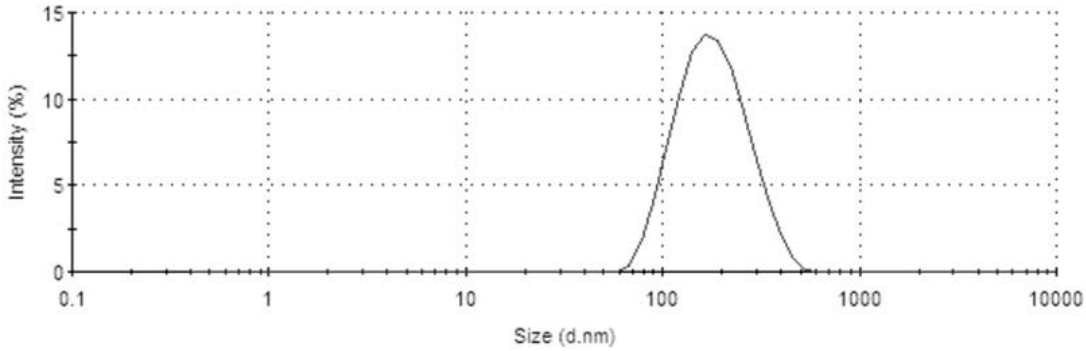


Figure 17 Size distribution of the extract-loaded niosomes



(a)



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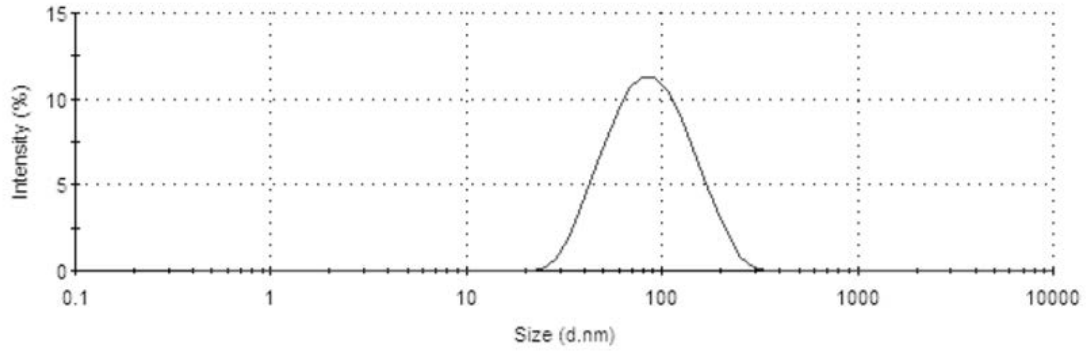


(c)

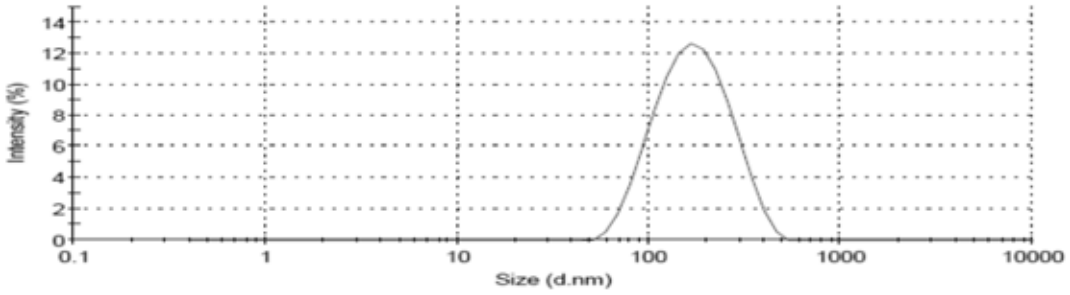
Figure 18 Size distribution of the extract-loaded nanoemulsions: NE1 (a), NE2 (b) and NE3 (c)

3.3.4 Elasticity determination of elastic liposomes

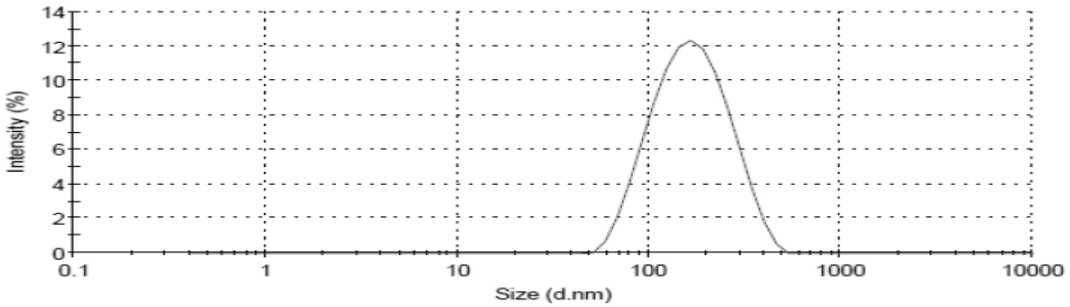
After extrusion of the elastic liposomes through the 50 nm filter membrane, their average diameter and entrapment efficiency were similar to the initial elastic liposomes (Table 14 and Figure 19). It could confirm elasticity of the elastic liposomes that can squeeze through tiny membrane without breaking (Cevc, 1996).

Table 14 Size, size distribution and entrapment efficiency of the extract-loaded elastic liposomes before and after extrusion (n = 3, mean ± SD)

Elastic liposomes	Size (nm)	Polydispersity index	% Entrapment efficiency
After preparation	152.46 ± 0.66	0.120 - 0.163	60.76 ± 0.39
After extrusion	150.27 ± 0.10	0.113 - 0.170	60.15 ± 0.20



(a)



(b)

Figure 19 Size distributions of the extract-loaded elastic liposomes before (a) and after (b) extrusion through 50 nm membrane

3.3.5 Entrapment efficiency of the extract in conventional liposomes, elastic liposomes and niosomes

In this study, entrapment efficiency of the extract in vesicular systems was investigated only by DPPH inhibition assay. The entrapment efficiencies of conventional liposomes, elastic liposomes and niosomes were 53.31 ± 0.50 %, 60.76 ± 0.39 % and 58.91 ± 0.91 %, respectively. The results were similar to those of *P. emblica* extract liposomes and niosomes of other studies which were determined by ultracentrifugation. For examples, Muntamon Utamo et al. (2011) showed that entrapment efficiency of *P. emblica* extract liposomes varied from 49 % to 95 % (assayed by Folin-Ciocalteu colorimetric method). Boontarika Kawswang et al. (2009) reported entrapment efficiency of *P. emblica* extract in niosomes at 64 - 89 % (assayed by …). In the present study, elastic liposomes and niosomes showed significantly higher entrapment efficiency than conventional liposomes ($p < 0.05$). However, the entrapment efficiencies of elastic liposomes and niosomes were insignificantly different. Elastic liposomes contained a Span® 80, which is a membrane destabilizer (Hofer et al., 2000). During breaking and reforming of phospholipid bilayers, the content of vesicles is exchanged with the dispersion medium. At the end of preparation, the extract was inside the elastic liposomes resulting in higher entrapment efficiency. High entrapment efficiency of transfersomes has also been reported by Hofer et al. (2000) (84 and 80% entrapment of interferon and interleukin-2, respectively) and Alvi et al. (2011) (82% entrapment of 5-fluorouracil). For niosomal formulation, high entrapment efficiency was due to large vesicle sizes leading to large capacity for the entrapped extract.

3.3.6 Content of the extract in nanosystems

The extract content in nanosystems was analyzed by DPPH radical scavenging assay and tyrosinase inhibition assay except the vesicular systems. The results are shown in Tables 15 and 16. Similar results of the extract content in microemulsions and nanoemulsions were obtained from both assay methods. All nanosystem formulations provided nearly 100 percent of the labeled amount of the

extract. Therefore, all extract-loaded nanosystems could be prepared without the extract loss during their manufacturing process.

Table 15 Content and percent labeled amount of the extract in nanosystems analyzed by DPPH radical scavenging assay (n = 9, mean ± SD)

Formulations	Extract content (mg/mL)	% Labeled amount of extract
Conventional liposomes	5.11 ± 0.00	99.98 ± 0.02
Elastic liposomes	5.57 ± 0.01	99.90 ± 0.16
Niosomes	5.09 ± 0.02	99.67 ± 0.36
ME1	60.35 ± 0.20	99.25 ± 0.34
ME2	32.77 ± 0.15	99.90 ± 0.45
ME3	40.52 ± 0.16	99.31 ± 0.39
NE1	21.09 ± 0.01	99.94 ± 0.04
NE2	ND	ND
NE3	22.84 ± 0.61	98.45 ± 2.62

ND = not determined

Table 16 Content and percent labeled amount of the extract in nanosystems analyzed by tyrosinase inhibition assay (n = 9, mean ± SD)

Formulations	Extract content (mg/mL)	% Labeled amount of extract
ME1	60.43 ± 0.28	99.39 ± 0.46
ME2	32.44 ± 0.45	99.02 ± 1.53
ME3	40.64 ± 0.15	99.80 ± 0.22
NE1	20.90 ± 0.19	99.75 ± 0.33
NE2	ND	ND
NE3	22.62 ± 0.33	97.31 ± 2.04

ND = not determined

3.3.7 Stability study of nanosystems

3.3.7.1 Physical stability

Physical stability of all nanosystems was investigated under the conditions as mentioned in Chapter III, 3.4.6. Results are shown as follows.

3.3.7.1.1 Conventional liposomes

The conventional liposomes were stored at 4 ºC for 8 weeks. The appearance and particle size of the vesicles are presented in Figures 20 and 21, respectively. After storage at 4 ºC for 8 weeks, no sedimentation was observed and pH of liposomal dispersion was constant at 5.49 ± 0.03. The average size of the conventional liposomes gradually increased with time. However, their average sizes remained within the nanometer range. Figure 22 showed that the entrapment efficiency of conventional liposomes decreased from 53.31 ± 0.50 % to 49.35 ± 0.87 % after 8 weeks. The extract leakage of less than 10 % of the initial load was well within the limits (Patel et al., 2010). Therefore, the conventional liposomes were reasonably stable in terms of aggregation and fusion over the studied storage period.

(a) (b)

Figure 20 Physical appearance of the extract-loaded conventional liposomes after preparation (a) and after storage at 4 ºC for 8 weeks (b)

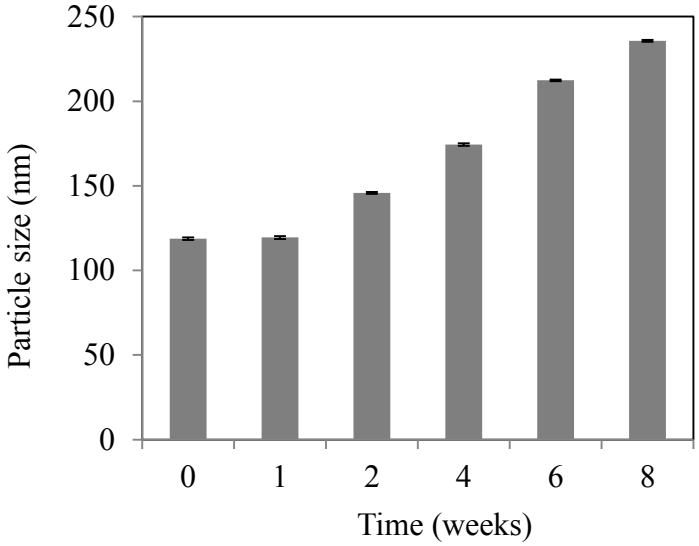


Figure 21 Particle size of the extract-loaded conventional liposomes under storage at 4 ºC for 8 weeks (n = 3)

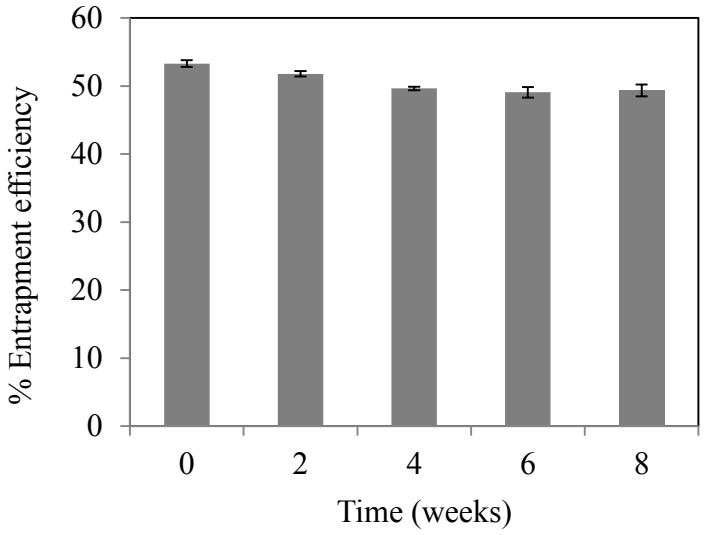


Figure 22 Entrapment efficiency of the extract in the conventional liposomes under storage at 4 ºC for 8 weeks (n = 3)

3.3.7.1.2 Elastic liposomes

The elastic liposomes were stored at 4 ºC for 8 weeks. No sedimentation was found after 8 weeks (Figure 23). The pH value of the dispersion did not change (5.49 ± 0.02) during this period. Its particle size slightly increased with

time (Figure 24). However, changes in size of the elastic liposomes were less than that of the conventional liposomes.

(a) (b)

Figure 23 Physical appearance of the extract-loaded elastic liposomes after preparation (a) and after storage at 4 ºC for 8 weeks (b)

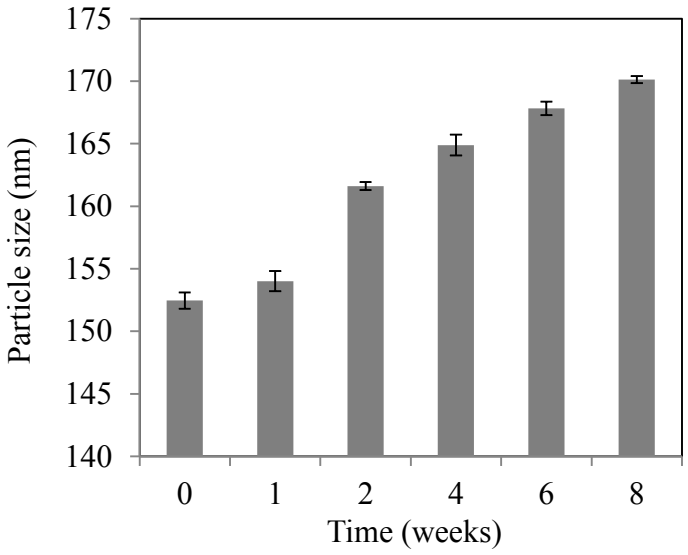


Figure 24 Particle size of the extract-loaded elastic liposomes under storage at 4 ºC for 8 weeks (n = 3)

In addition, entrapment efficiency of elastic liposomes decreased from 59.25 ± 0.50 % to 54.29 ± 0.453 % after storage for 4 weeks and remained unchanged until 8 weeks (Figure 25).

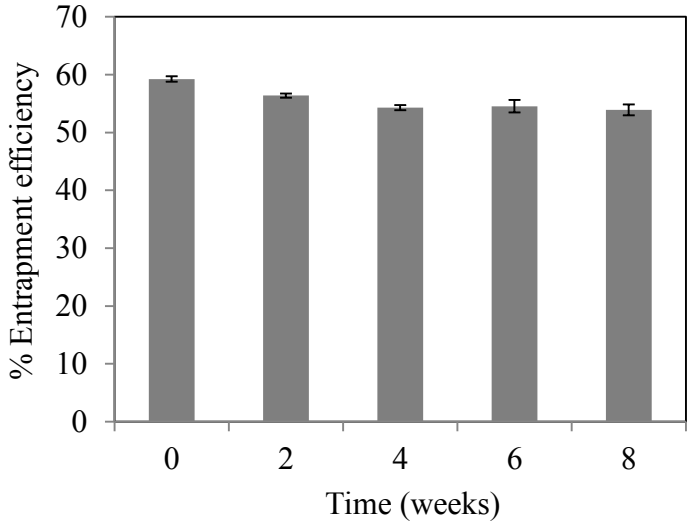


Figure 25 Entrapment efficiency of the extract in the elastic liposomes under storage at 4 ºC for 8 weeks (n = 3)

3.3.7.1.3 Niosomes

To evaluate the stability, niosomes were stored at room temperature for 8 weeks. Within 8 weeks, the niosomes were more opaque with an increase in particle size (Figures 26 and 27). However, no sedimentation was observed and pH value of dispersion unchanged (5.50 ± 0.03). Their average sizes were within the nanometer range.

(a) (b)

Figure 26 Physical appearance of the extract-loaded niosomes after preparation (a) and after storage at room temperature for 8 weeks (b)

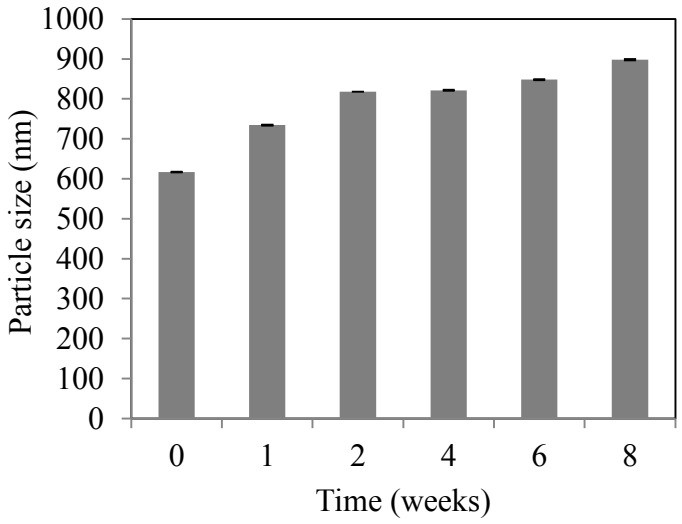


Figure 27 Particle size of the extract-loaded niosomes under storage at room temperature for 8 weeks (n = 3)

Figure 28 showed that entrapment efficiency of niosomes decreased from 58.34 ± 0.319 % to 55.80 ± 0.479 % after storage for 4 weeks and remained unchanged until 8 weeks.

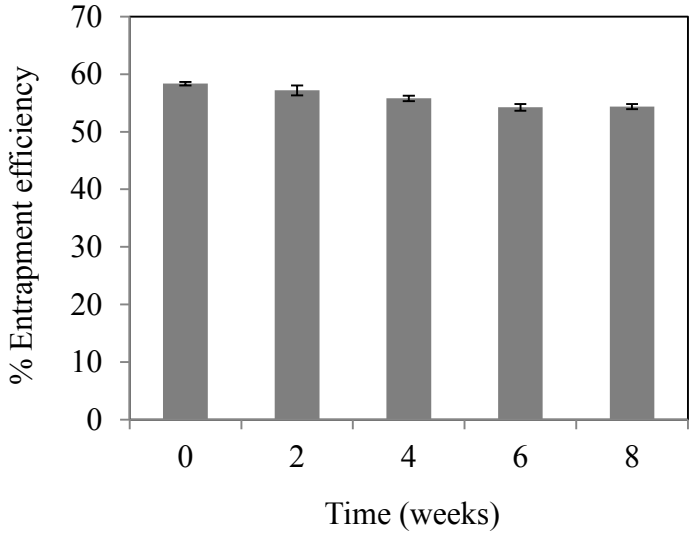


Figure 28 Entrapment efficiency of the extract in the niosomes under storage at 4 ºC for 8 weeks (n = 3)

A decrease in entrapment efficiency of conventional liposomes, elastic liposomes and niosomes indicated the leakage of vesicle membrane. This might be due to membrane fluidity of the vesicles. The change in entrapment efficiency was not significantly different among the vesicular systems.

3.3.7.1.4 Microemulsions

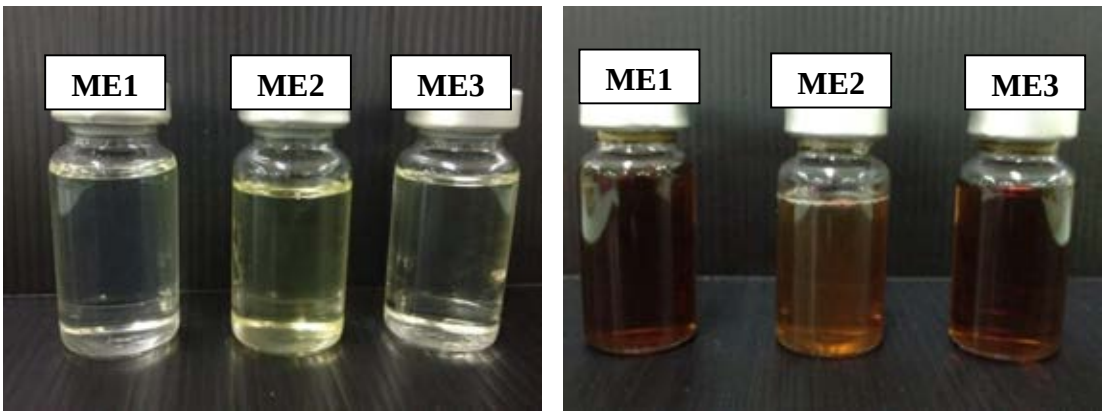
After storage of blank microemulsions and the extract-loaded microemulsions at room temperature for 8 weeks, the physical appearance of all microemulsions did not change in terms of color and transparency (Figure 29). Their pH value was constant at pH 5.43 - 5.53. Phase separation and extract precipitation were not found. These microemulsions were considerably physically stable.

3.3.7.1.5 Nanoemulsions

Physical stability of the blank nanoemulsions and the extract-loaded nanoemulsions was evaluated at accelerated condition and at room temperature. At accelerated condition, the nanoemulsions were subjected to heating-cooling cycle. The formulations that were stable at this condition were subjected to the freeze-thaw cycle test. Their physical appearance after heating-cooling cycle test is shown in Figure 30. The result showed that the formulation NE3 was the most stable. It remained unchanged in terms of appearance and pH (constant at 5.5) after the 4^{th} cycle. After the 5^{th} cycle, the formulation NE3 was more opaque. However, no extract precipitation was found and its particle size did not change (Figure 31). The formulation NE1 was more opaque and the particle size increased as number of heating-cooling cycles increased. However, phase separation and extract precipitation were not found. The formulation NE2 was the least stable. Creaming and cracking were observed in the formulation NE2 after passing the 3^{th} cycle and the 6^{th} cycle, respectively. Larger particle size of the formulation NE2 leaded to merging of droplets in this formulation. As a result, the formulations NE1 and NE3 were subjected to the further freeze-thaw cycle test.

For the freeze-thaw cycle test, the result showed that no phase separation and sedimentation were observed in the formulations NE1 and NE3 at the end of study (Figure 32). Particle size of the formulation NE1 significantly increased ($p < 0.05$) while that of the formulation NE3 did not change (Figure 33). However, the particle size of both nanoemulsions was still in the nanometer range. Thus, the formulations NE1 and NE3 were considered physically stable.

(a) After preparation

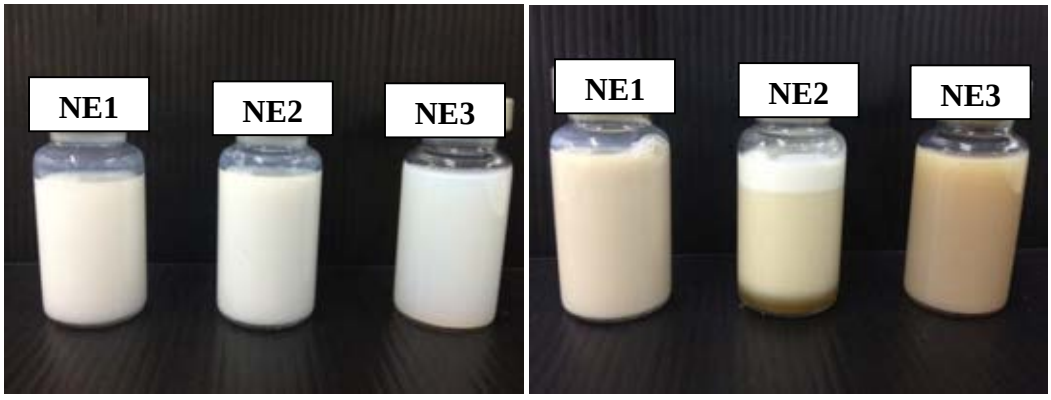


(b) After storage at room temperature for 8 weeks

Figure 29 Physical appearance of blank microemulsions (left) and the extract-loaded microemulsions (right) after preparation and after storage at room temperature for 8 weeks

(a) After preparation

(b) After the 3th heating-cooling cycle



(c) After the 6th heating-cooling cycle

Figure 30 Physical appearance of blank nanoemulsions (left) and the extract-loaded nanoemulsions (right) after preparation and after passing heating-cooling cycle

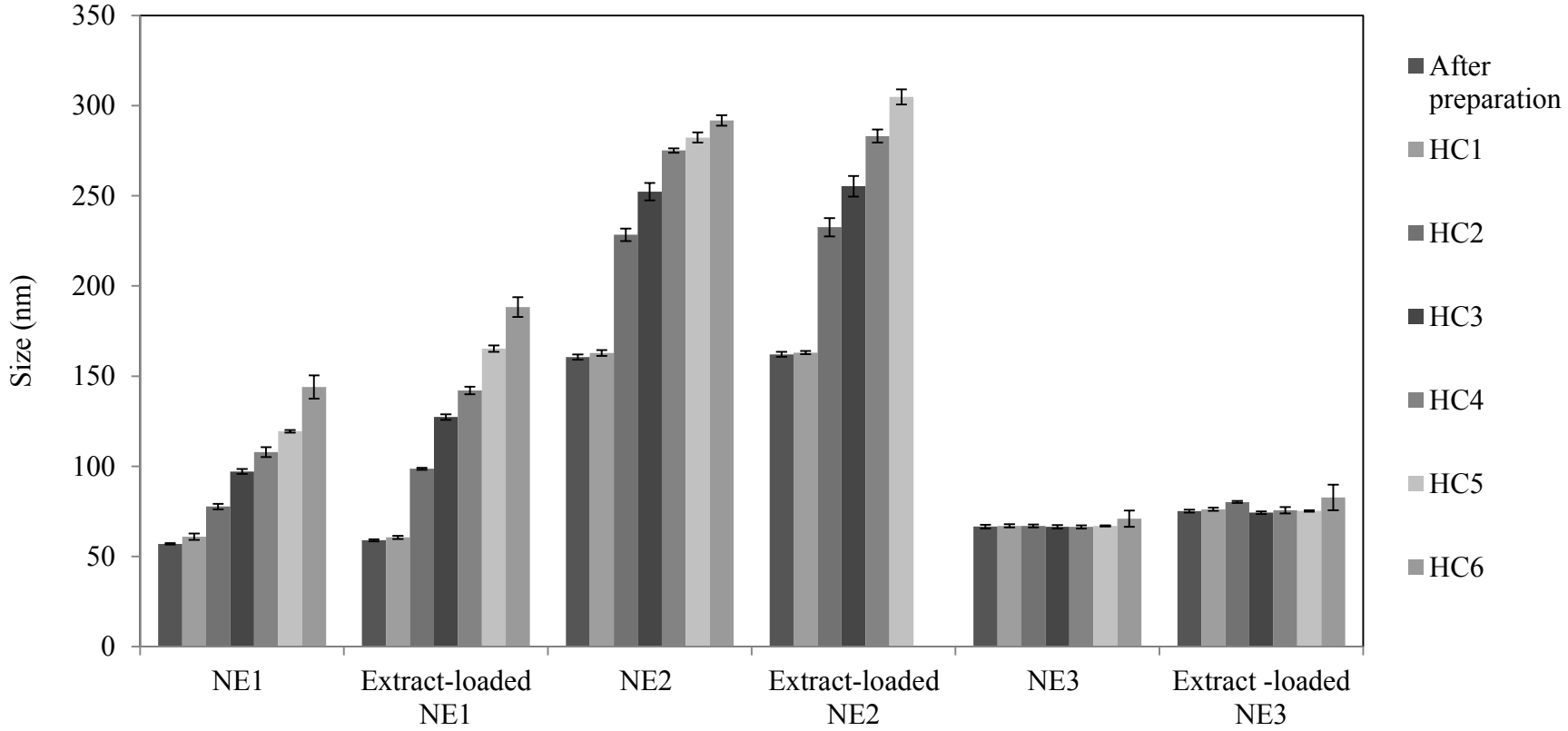
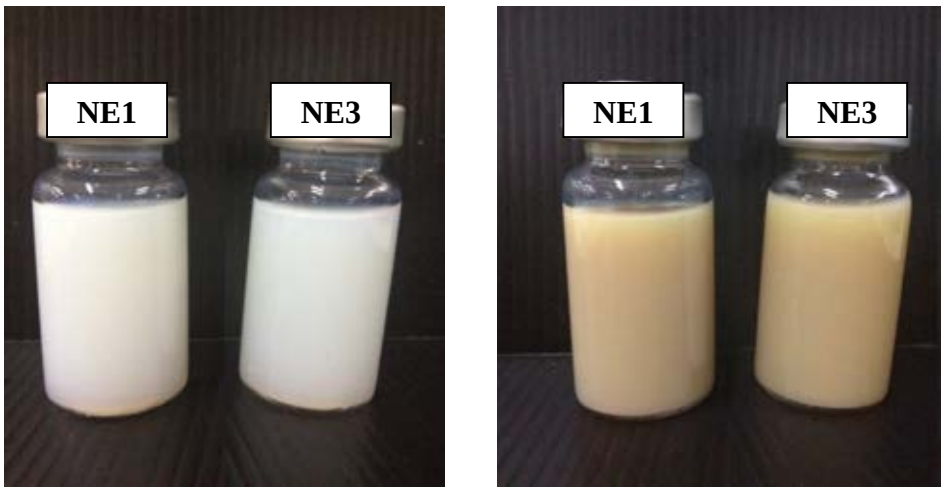
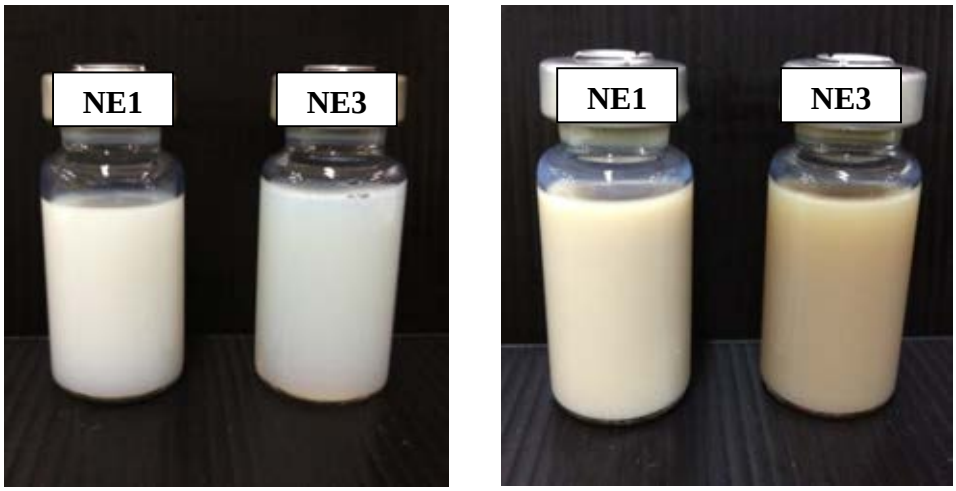


Figure 31 Particle size of blank and the extract-loaded nanoemulsions after preparation and after passing heating-cooling cycle (n = 3)



(a) After preparation



(b) After the 3rd freeze-thaw cycle

Figure 32 Physical appearance of blank nanoemulsions (left) and the extract-loaded nanoemulsions (right) after preparation and after passing freeze-thaw cycle

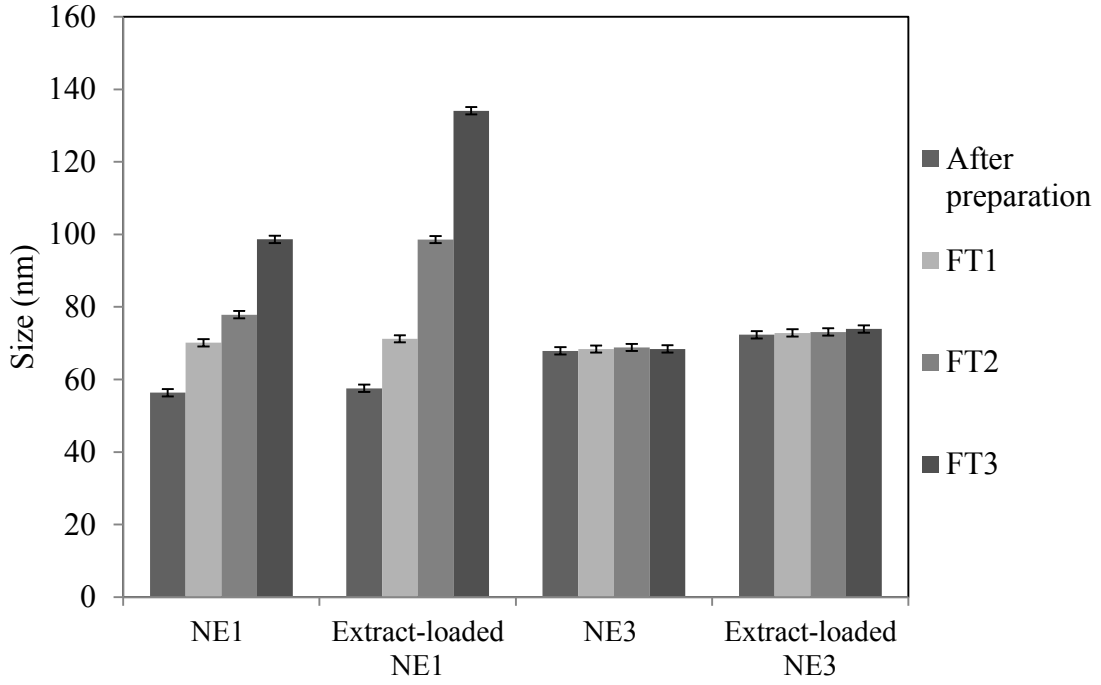
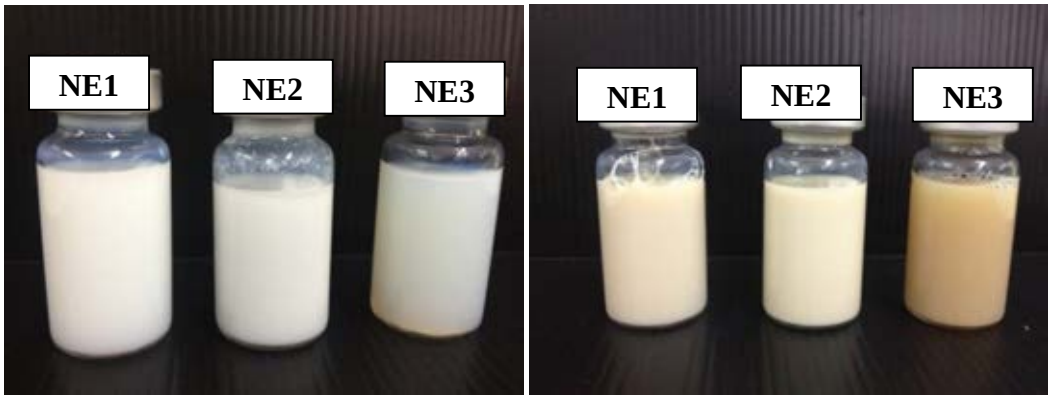


Figure 33 Particle size of blank and the extract loaded nanoemulsions after preparation and after passing freeze-thaw cycle (n = 3)

Under storage at room temperature for 8 weeks, physical appearance of nanoemulsions is shown in Figure 34. For the formulation NE2, creaming and phase separation were found for both blank and the extract-loaded nanoemulsions at the 3th week and the 6th week, respectively. Instability of NE2 might be due to its large particle size, which was easy to aggregate. Their particle size significantly increased since the 1st week. The formulations NE1 and NE3 remained unchanged in terms of appearance and pH (constant at 5.5). Phase separation and extract precipitation were not found. But their particle size significantly increased (Figure 35). Particle size of both blank and extract-loaded NE1 significantly increased since the 1st week ($p < 0.05$). Particle size of blank and extract-loaded NE3 significantly increased at the 8th and 4th week, respectively ($p < 0.05$). This indicated that the extract might affect the aggregation of nanoemulsions. However, the particle size of the formulation NE1 and NE3 remained within the range of nanometers. Better stability of the formulation NE1 and NE3 may be due to the optimal compositions of oil and surfactants. The optimum curvature of the interfacial layer by surfactant caused

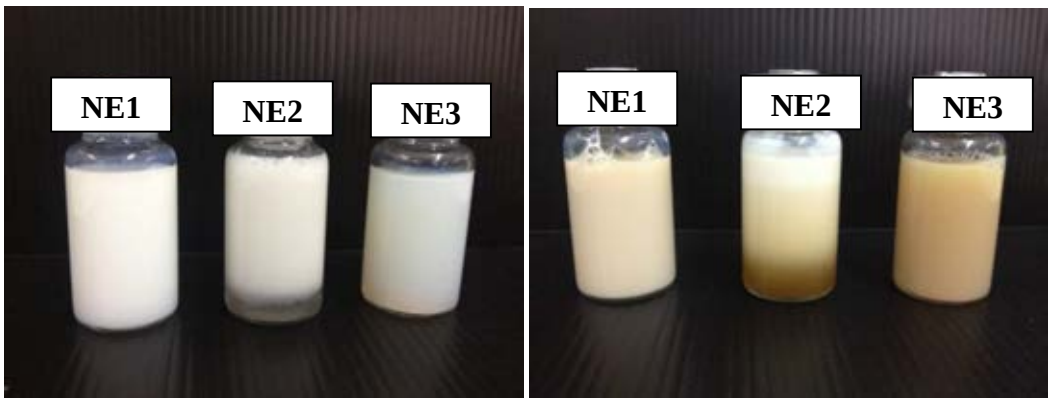
increasing the repulsive interactions between the droplets (Rao and McClements, 2010). Therefore, the formulations NE1 and NE3 were selected for further chemical stability test.

(a) After preparation

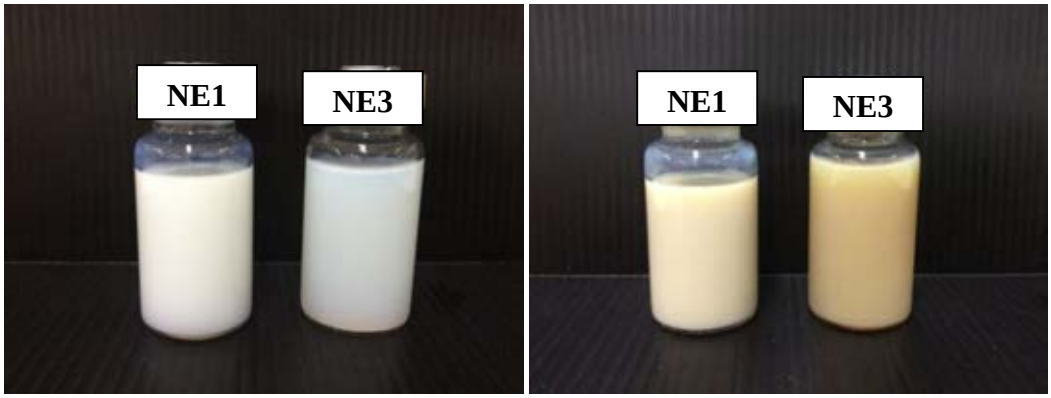


(b) After storage at room temperature for 3 weeks

Figure 34 Physical appearance of blank nanoemulsions (left) and the extract-loaded nanoemulsions (right) after preparation and after storage at room temperature



(c) After storage at room temperature for 6 weeks



(d) After storage at room temperature for 8 weeks

Figure 34 Physical appearance of blank nanoemulsions (left) and the extract-loaded nanoemulsions (right) after preparation and after storage at room temperature (continued)

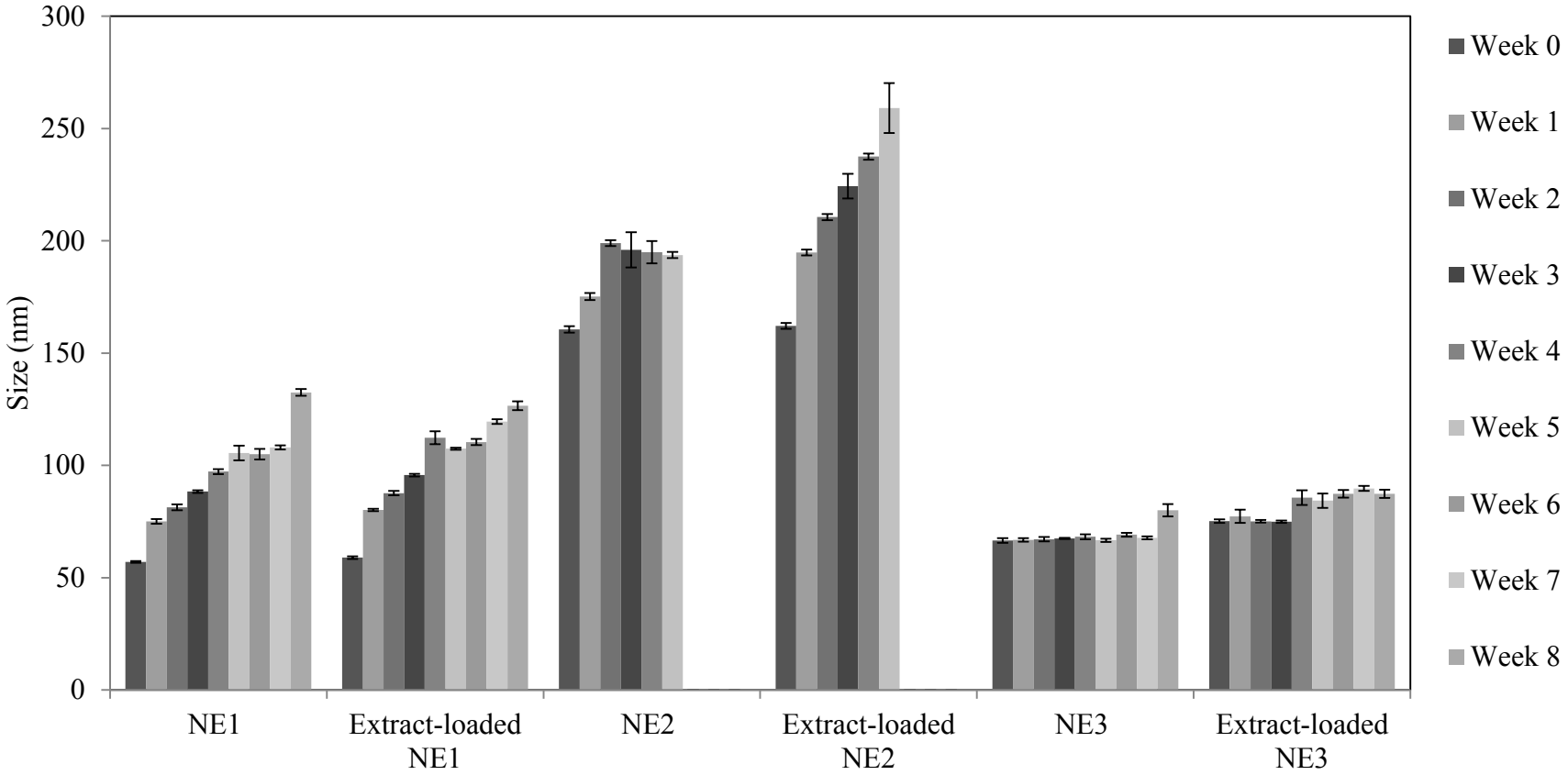


Figure 35 Particle size of blank and extract-loaded nanoemulsions stored at room temperature at any time interval (n = 3)

3.3.7.2 Biological stability

To evaluate biological stability of the extract in nanosystems, the conventional liposomes and elastic liposomes were stored at 4 ºC for 8 weeks. Niosomes, microemulsions and nanoemulsions were kept at room temperature for 8 weeks. Figure 36 shows that the percentage of remaining extract in conventional liposomes, elastic liposomes and niosomes insignificantly changed after 8 weeks. This indicated that the extract was still biologically stable at 4 ºC (for both liposomes) and at room temperature (for niosomes) for at least 8 weeks although it leaked out of the vesicles.

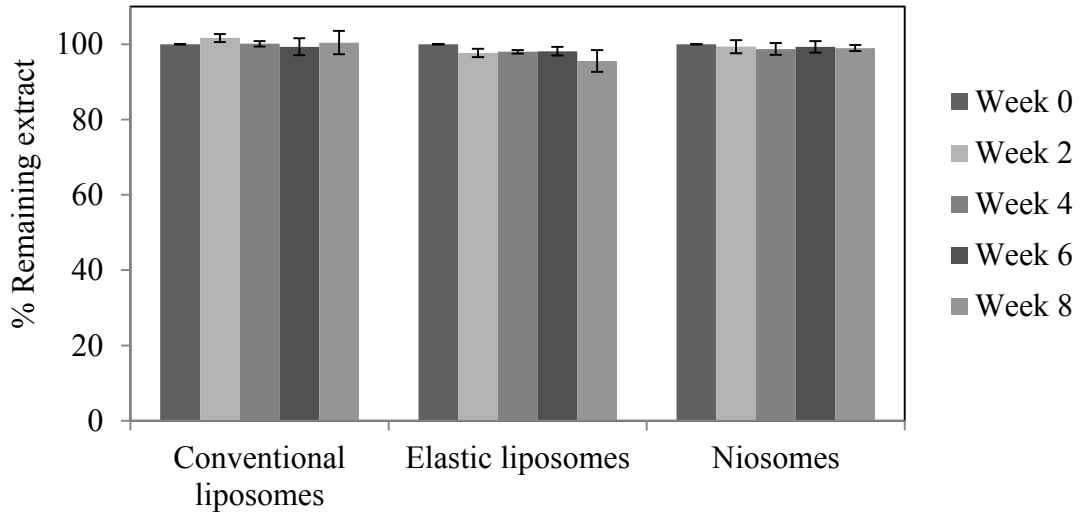


Figure 36 Percentage of remaining extract in conventional liposomes, elastic liposomes and niosomes under storage for 8 weeks investigated by DPPH inhibition activity (n = 3)

The percentages of remaining extract in microemulsions after storage at room temperature investigated by DPPH inhibition and tyrosinase inhibition activities are shown in Figures 37 and 38, respectively. There was no significant change in both biological activities of the extract in all formulations during the stability test for 8 weeks. The results indicated that the extract in microemulsions was biologically stable at room temperature for at least 8 weeks.

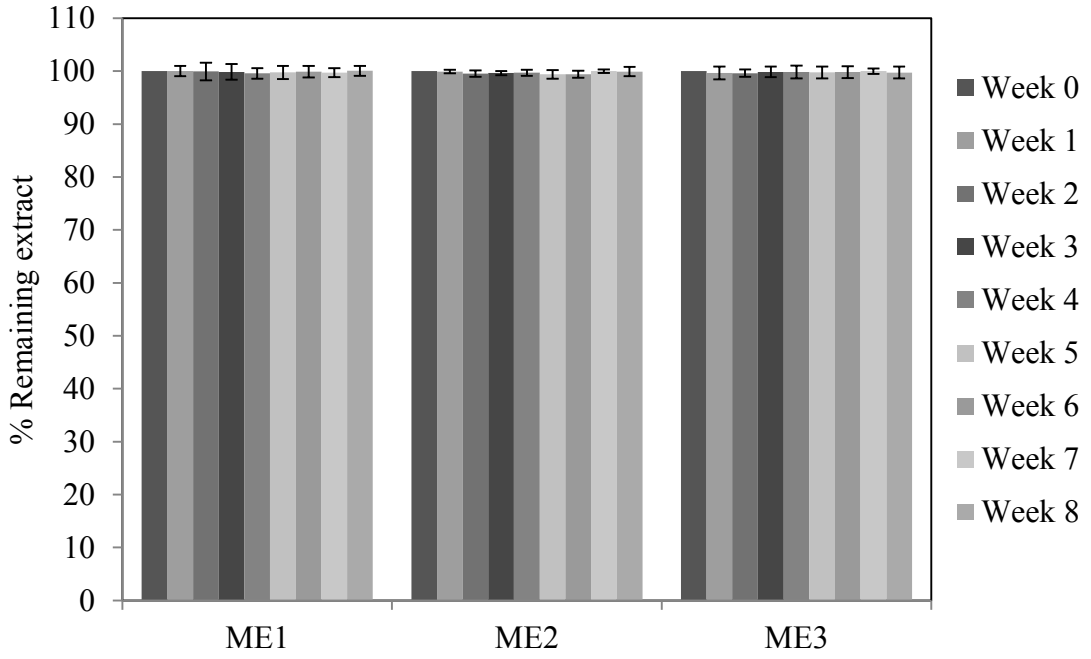


Figure 37 Percentage of remaining extract in microemulsions after storage at room temperature investigated by DPPH inhibition activity (n = 3)

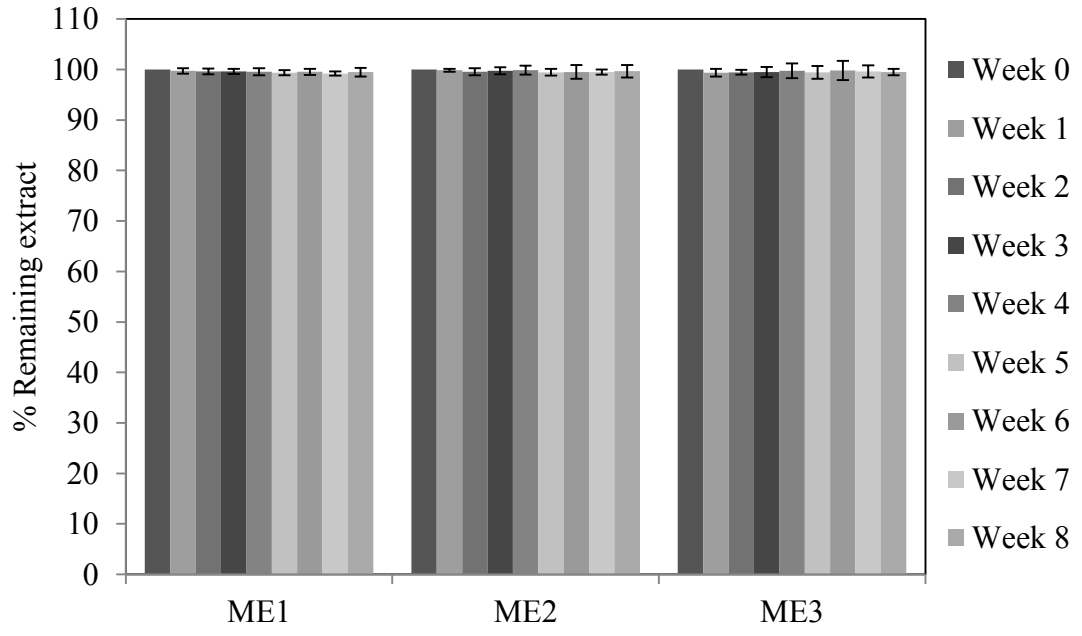


Figure 38 Percentage of remaining extract in microemulsions after storage at room temperature investigated by tyrosinase inhibition activity (n = 3)

For nanoemulsions, only the formulations NE1 and NE3 which passed physical stability test, were determined for their biological stability. The remaining extract in nanoemulsions was analyzed by DPPH radical scavenging and tyrosinase inhibition activities. The results are shown in Figures 39 and 40, respectively. After storage at room temperature for 8 weeks, both biological activities of the extract did not significantly change in all formulations. The result indicated that the extract in nanoemulsions was biologically stable at room temperature for at least 8 weeks.

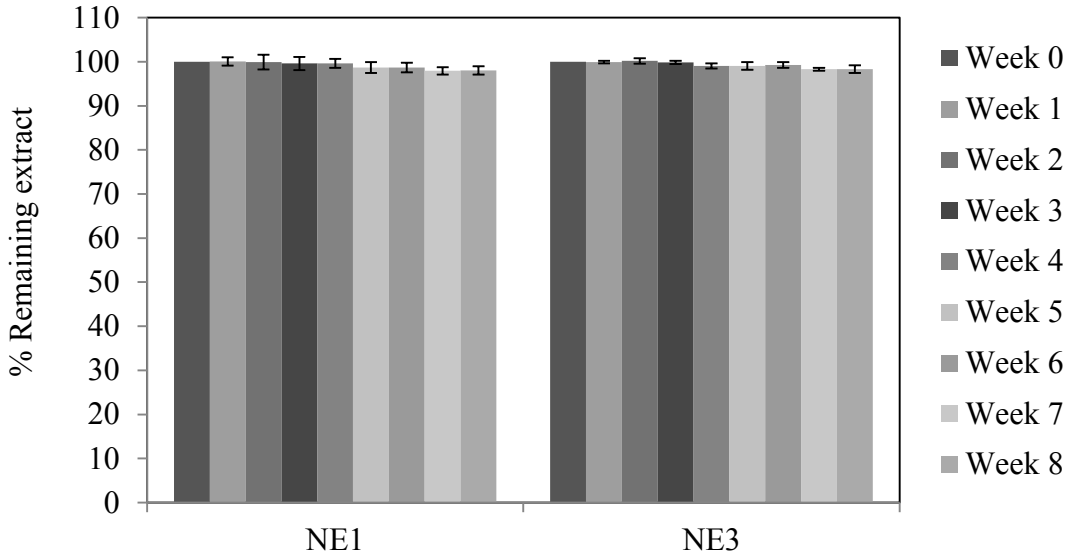


Figure 39 Percentage of remaining extract in nanoemulsions after storage at room temperature investigated by DPPH inhibition activity (n = 3)

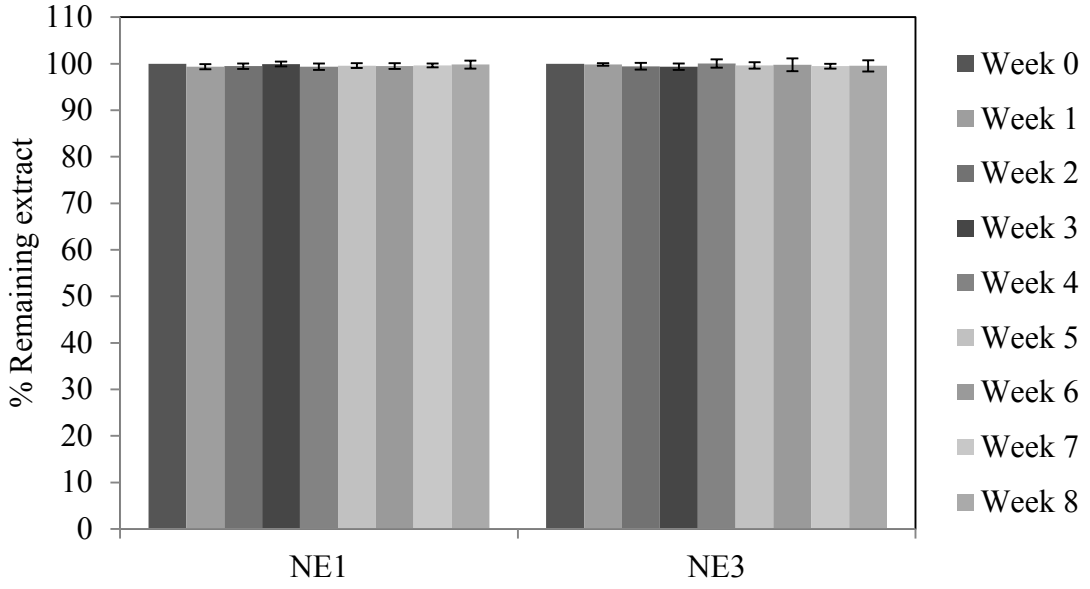


Figure 40 Percentage of remaining extract in nanoemulsions after storage at room temperature investigated by tyrosinase inhibition activity (n = 3)

The extract-loaded conventional liposomes, elastic liposomes, niosomes, ME1, ME2, ME3, NE1 and NE3 were biologically stable formulations. This might be because the extract was stable in a citrate buffer pH 5.5. This could be supported by the study of นิริน อุดมสม และคณะ (2548) who found that polyphenol content of *P. emblica* extract was stable during storage at pH 5.5 comparing to pH 3.5, 7.4 and 9. Moreover, Pornpen Kongaimpitee (2007) also found that the chemical stability investigated by total phenolic content of *P. emblica* extract was better in buffer solution pH 5.5 than in buffer solution pH 7.4. As the results, the conventional liposomes, elastic liposomes, niosomes, ME1, ME2, ME3, NE1 and NE3 were used for further *in vitro* release and *in vitro* skin permeation studies.

3.4 *In vitro* release study of the nanosystems

In vitro release study was performed on modified Franz-diffusion cell. Eight formulations of conventional liposomes, elastic liposomes, niosomes, ME1, ME2, ME3, NE1 and NE3 selected from 3.3.6 were investigated. Citrate buffer pH 5.5 was tested as a control for microemulsions and nanoemulsions. The solution of citrate buffer

pH 5.5:PG (80:20) was tested as a control for conventional liposomes and niosomes. The solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) was also tested as a control for elastic liposomes. All nanosystems and controls contained 90% saturated concentration of the extract.

The extract release profiles of all formulations and control solutions are shown in Figures 41 and 42. When the extract in a formulation was exposed to the receptor medium, it diffused out of the formulations. The extract in all formulations was released slowly compared to that from their controls. The diffusions of the extract from control solutions were nearly completed (> 90 %) within 6 hours. The vesicular systems gradually released the extract 14 – 21 % over 24 hours. Although the vesicular systems had the unentrapped extract about 39 – 47 %, the free extract could not be completely released from the formulations. This might be because of the extract-membrane interaction which should be further studied. However, many researchers also reported similar slowly release of various drugs from vesicles, for example, 5-retinoic acid (Montenegro et al., 1996) and carboxyfluorescein (Yoshioka et al., 1994). Among vesicular systems, elastic liposomes could release the most extract followed by conventional liposomes and niosomes. There was no significant difference between percent the extract release of conventional liposomes and niosomes. A higher release of elastic liposomes could be attributed to the higher membrane fluidity resulted from the incorporation of an edge activator.

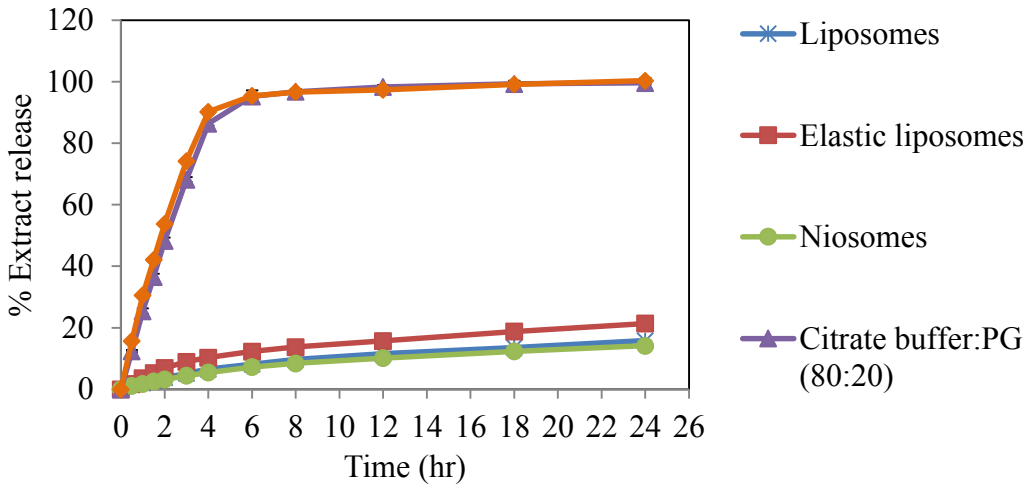


Figure 41 The extract release profiles of the vesicular systems and their controls (n = 3)

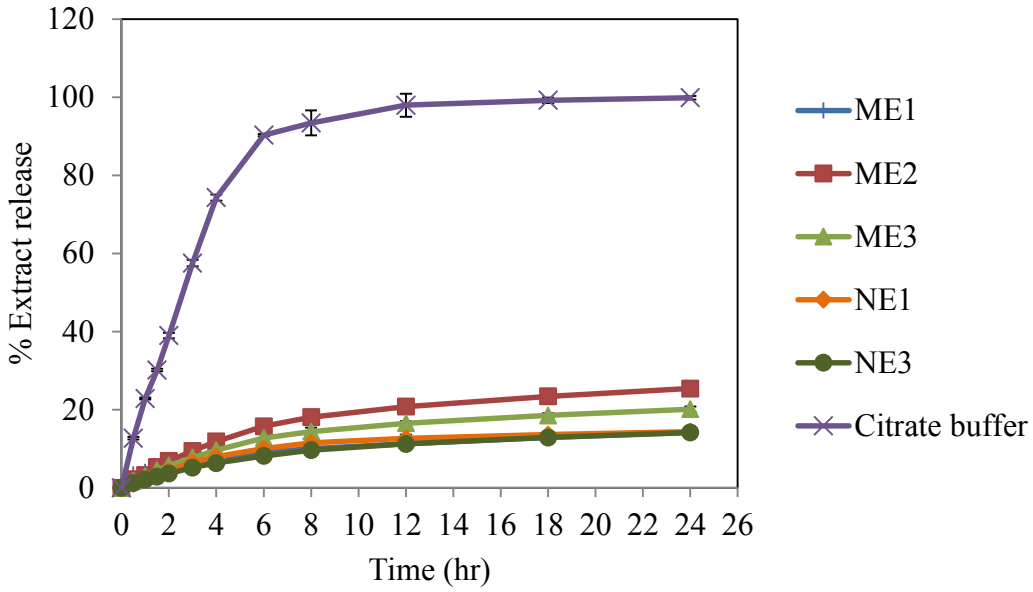


Figure 42 The extract release profiles of microemulsions, nanoemulsions and their controls (n = 3)

For microemulsions and nanoemulsions, the slow release of the extract was similar to that of the vesicular systems. The 14 - 25 % of loading amount was released from these formulations at 24 hours. Even larger amounts of the extract could be incorporated in microemulsions and nanoemulsions in comparison with citrate buffer pH 5.5. The extract seemed to have more affinity to the formulations. Among microemulsions, the formulation ME2 exhibited the maximum release of the extract followed by the formulation ME3 and ME1, respectively. In addition, the release of extract from the formulation NE1 was higher than that of NE3. The release of the extract from microemulsions and nanoemulsions seemed to follow the trend of their solubilities. Higher solubility of the extract in the formulations, lower the extract releasing out. Higher solubility of the extract in the formulation implied that the extract has higher affinity to formulations. Consequently, it is reasonable that the extract was likely to retain in the microemulsions and nanoemulsions than diffusing to a lower solubility medium such a citrate buffer pH 5.5.

3.5 Permeation study of the nanosystems

In vitro skin permeation study of the extract in the nanosystems was studied to find the suitable delivery systems for the *P. emblica* extract. The study was performed on modified Franz diffusion cell using a new born pig skin as the model membrane (Schmook et al., 2001). Conventional liposomes, elastic liposomes, niosomes, ME1, ME2, ME3, NE1 and NE3 selected from 3.2.6 were investigated. The 90% saturated concentration of the extract was incorporated to obtain equal thermodynamic activity and maximum flux across a membrane for all formulations (Higuchi, 1960).

The percentage of the extract found in the receptor chamber, skin and donor part after 24 hours of the study are presented in Table 17. The mass balance was within the range of 88 - 104 %. The good recovery indicated the accuracy of the studies. Data showed that the most amount of the extract was in the donor part, some was retained in skin and some could pass through skin to the receptor chamber.

Table 17 Percentage of the extract analyzed in each part after 24 hours of permeation study and % mass balance (n = 6, mean ± SD)

Formulations	% Extract analyzed after 24 hours			% Mass balance
	Receptor chamber	Skin	Donor part	
Conventional liposomes	1.619 ± 0.235	3.781 ± 0.142	85.897 ± 2.022	95.422 ± 4.983
Elastic liposomes	2.229 ± 0.102	4.420 ± 0.218	83.037 ± 1.724	89.686 ± 1.846
Niosomes	1.193 ± 0.160	3.076 ± 0.191	84.880 ± 2.784	89.149 ± 2.691
ME1	0.290 ± 0.024	0.754 ± 0.040	91.954 ± 3.007	92.997 ± 2.975
ME2	1.769 ± 0.158	2.110 ± 0.261	89.306 ± 1.494	93.186 ± 1.348
ME3	3.062 ± 0.143	5.674 ± 0.156	92.259 ± 2.539	100.996 ± 2.685
NE1	4.046 ± 0.468	9.771 ± 0.835	90.190 ± 2.607	104.005 ± 2.874
NE3	1.564 ± 0.046	2.563 ± 0.066	91.970 ± 3.562	96.097 ± 3.514
Citrate buffer pH 5.5	0.098 ± 0.021	0.260 ± 0.099	90.960 ± 2.191	91.233 ± 2.201
Citrate buffer pH 5.5:PG (80:20)	0.205 ± 0.015	0.573 ± 0.100	91.551 ± 3.126	92.328 ± 3.125
Citrate buffer pH 5.5:PG: ethanol (73:20:7)	0.408 ± 0.031	0.731 ± 0.096	87.717 ± 1.709	88.857 ± 1.733

The permeation profiles of the extract loaded in various nanosystems are depicted in Figures 43 and 44. The plots clearly showed permeation of the extract from all formulations and controls through the skin membrane.

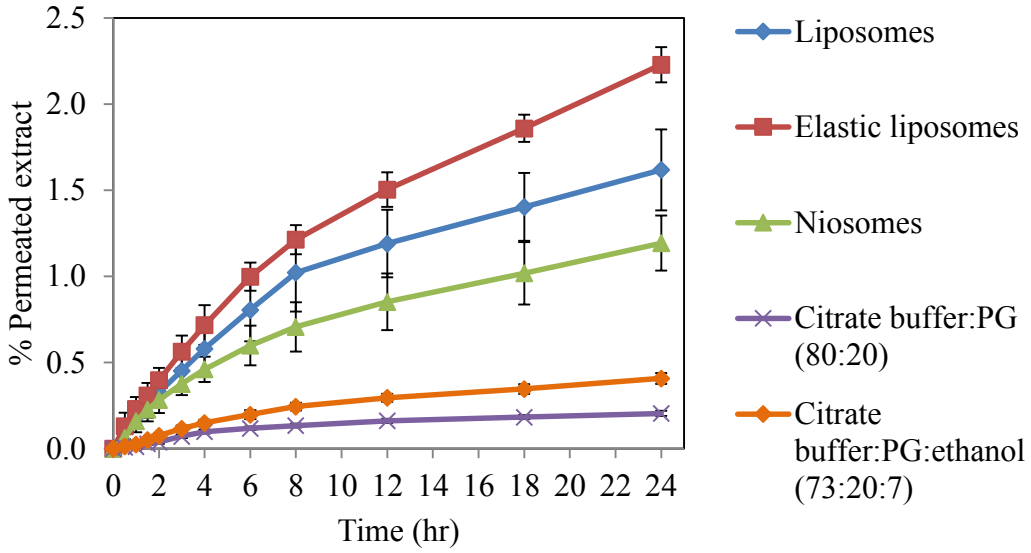


Figure 43 *In vitro* extract permeation profile of the vesicular systems and their controls (n=6)

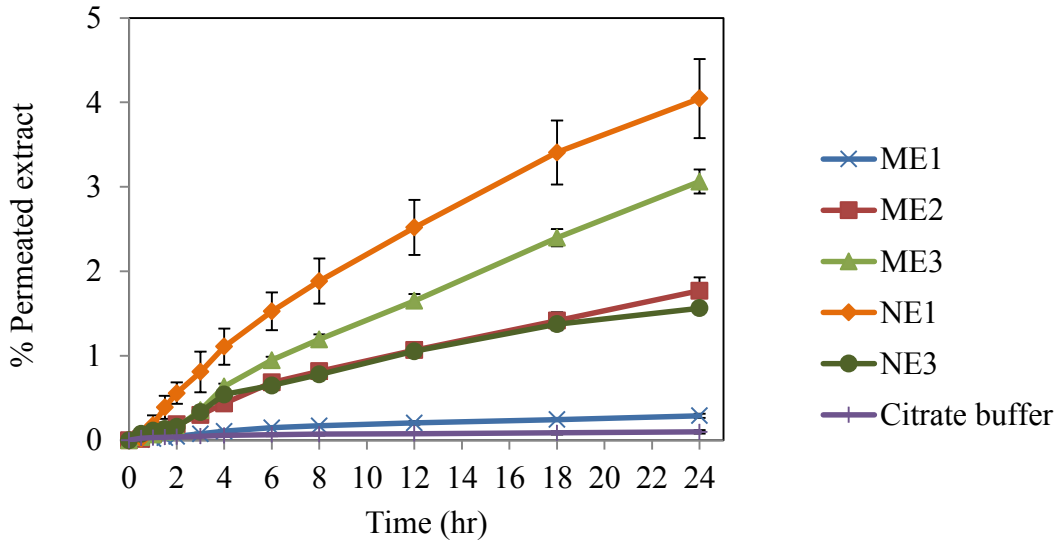


Figure 44 *In vitro* extract permeation profile of microemulsions, nanoemulsions and their control (n=6)

Although some data in the receptor compartment at the beginning of permeation studies were below lower limit of quantification (LLOQ) (see Appendix C and D). The results were calculated for comparison purpose. Tables 18 and 19 showed that all parameters of conventional liposomes and niosomes were significantly higher than those of the solution of citrate buffer pH 5.5:PG (80:20) ($p < 0.05$). In the same way, Table 20 showed that these parameters of elastic liposomes was significantly higher in comparison with the solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) ($p < 0.05$). The results indicated that the vesicular systems could improve skin delivery of the extract over their controls. This result was because of their vesicular structure and skin penetration enhancement of their composition. The similar result was reported by Garg et al. (2008). They showed that flux, RF and Q_s (%) of rizatriptan in elastic liposomes and conventional liposomes were higher than its PBS solution. El Maghraby et al. (1999) concluded elastic and conventional liposomes showed better the skin delivery of oestradiol through human skin comparing with an aqueous solution. In the same way, niosomes could improve the extent of N-acetyl glucosamine in the skin compared to hydroalcoholic solution (Shatalebi et al., 2010).

Table 18 Permeation parameters of the extract from conventional liposomes and its control (n = 6, mean ± SD)

Parameters	Conventional liposomes	Citrate buffer pH 5.5:PG (80:20)	*p*-value
Flux	1.140 ± 0.211	0.118 ± 0.032	0.000*
$P_s \times 10^{-4}$	2.232 ± 0.413	0.231 ± 0.063	0.000*
Q_s (%)	3.781 ± 0.142	0.573 ± 0.100	0.000*
Q_{24}(%)	1.619 ± 0.235	0.205 ± 0.015	0.000*
EF	9.654 ± 1.788	1.000	0.024*
EF of % Q_s	6.603 ± 0.249	1.000	0.000*
EF of % Q_{24}	7.911 ± 1.149	1.000	0.023*
RF	9.652 ± 1.787	1.000	0.024*

* Significant at $p < 0.05$

Table 19 Permeation parameters of the extract from niosomes and its control (n = 6, mean ± SD)

Parameters	Niosomes	Citrate buffer pH 5.5:PG (80:20)	*p*-value
Flux	0.777 ± 0.227	0.118 ± 0.032	0.001*
$P_s \times 10^{-4}$	1.528 ± 0.446	0.231 ± 0.063	0.001*
Q_s (%)	3.076 ± 0.191	0.573 ± 0.100	0.000*
Q_{24} (%)	1.193 ± 0.160	0.205 ± 0.015	0.000*
EF	6.610 ± 1.927	1.000	0.000*
EF of % Q_s	5.372 ± 0.333	1.000	0.000*
EF of % Q_{24}	5.831 ± 0.782	1.000	0.000*
RF	6.580 ± 1.919	1.000	0.000*

* Significant at $p < 0.05$

Table 20 Permeation parameters of the extract from elastic liposomes and its control (n = 6, mean ± SD)

Parameters	Elastic liposomes	Citrate buffer pH 5.5:PG:ethanol (73:20:7)	*p*-value
Flux	1.641 ± 0.142	0.279 ± 0.029	0.000*
$P_s \times 10^{-4}$	2.945 ± 0.255	0.501 ± 0.052	0.000*
Q_s (%)	4.420 ± 0.218	0.731 ± 0.096	0.000*
Q_{24} (%)	2.229 ± 0.102	0.408 ± 0.031	0.000*
EF	5.881 ± 0.509	1.000	0.000*
EF of % Q_s	6.045 ± 0.298	1.000	0.000*
EF of % Q_{24}	5.464 ± 0.250	1.000	0.000*
RF	5.876 ± 0.508	1.000	0.000*

* Significant at $p < 0.05$

Among the vesicular systems, all permeation parameters in Table 21 were significantly different ($p < 0.05$). EF, EF of Q_{24} and RF of elastic liposomes were not significantly different from those of niosomes. As seen from EF, EF of % Q_s, EF of % Q_{24} and RF, the elastic liposomes could significantly enhance permeability coefficient, accumulated extract in skin, the extract permeation and relative flux less than conventional liposomes ($p < 0.05$). The reason for this result was that the permeation parameters of the extract in solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) were significantly higher than that of the solution of citrate buffer pH 5.5:PG (80:20) (Table 22). However, flux, P_s, Q_s (%) and Q_{24} (%) of elastic liposomes were significantly higher than those of liposomes and niosomes. This could imply that elastic liposomes were more effective than conventional liposomes and niosomes in skin delivery of the extract. This might be because the elastic liposomes can press through the pores in the stratum corneum and adsorb onto or fuse with the stratum corneum. The incorporation of surfactant or ethanol into the liposomes could also provide synergistic enhancement of the skin permeation (El Maghraby et al., 2000). In addition, the higher percentage of the released extract of elastic liposomes also provided higher concentration for penetration. The similar result was also reported by Trotta et al. (2004). The penetration of methotrexate across excised pig skin from elastic liposomes was found to be three-folds higher than that of conventional liposomes. In accordance with El Maghraby et al. (1999), the elastic vesicles composed of phosphatidylcholine with various edge activators could deliver higher oestradiol across human skin than conventional liposomes.

Considering microemulsions, Tables 23, 24 and 25 shows that all parameters were significantly higher than those of citrate buffer pH 5.5 ($p < 0.05$). Improvement in skin delivery of microemulsions over the control was due to skin penetration enhancement of their compositions. Surfactant, co-surfactant and penetration enhancer oils solubilized the skin lipid and then the extract could pass through skin easier. The result was in accordance to the previous report that showed the higher permeation of triptolide loaded microemulsions as compared to an aqueous solution of 20% propylene glycol (Chen et al., 2004).

Table 21 Permeation parameters of the extract from the vesicular systems (n = 6, mean ± SD)

Parameters	Liposomes	Elastic liposomes	Niosomes	*p*-value
Flux	1.140 ± 0.211	1.641 ± 0.142	0.777 ± 0.227	0.000*
$P_s \times 10^{-4}$	2.232 ± 0.413	2.945 ± 0.255	1.528 ± 0.446	0.000*
Q_s (%)	3.781 ± 0.142	4.420 ± 0.218	3.076 ± 0.191	0.000*
Q_{24} (%)	1.619 ± 0.235	2.229 ± 0.102	1.193 ± 0.160	0.000*
EF	9.654 ± 1.788	5.881 ± 0.509	6.610 ± 1.927	0.002*
EF of % Q_s	6.603 ± 0.249	6.045 ± 0.298	5.372 ± 0.333	0.000*
EF of % Q_{24}	7.911 ± 1.149	5.464 ± 0.250	5.831 ± 0.782	0.000*
RF	9.652 ± 1.787	5.876 ± 0.508	6.580 ± 1.919	0.002*

* Significant at $p < 0.05$

Table 22 Permeation parameters of the extract from the solution of citrate buffer pH 5.5:PG (80:20) and the solution of citrate buffer pH 5.5:PG:ethanol (73:20:7) (n = 6, mean ± SD)

Parameters	Citrate buffer pH 5.5:PG (80:20)	Citrate buffer pH 5.5:PG:ethanol (73:20:7)	*p*-value
Flux	0.118 ± 0.032	0.279 ± 0.029	0.000*
$P_s \times 10^{-4}$	0.231 ± 0.063	0.501 ± 0.052	0.000*
Q_s (%)	0.573 ± 0.100	0.731 ± 0.096	0.018*
Q_{24} (%)	0.205 ± 0.015	0.408 ± 0.031	0.000*

* Significant at $p < 0.05$

Table 26 shows that all permeation parameters were significantly different ($p < 0.05$) among microemulsions. The results showed that EF, EF of % Q_s, EF of % Q_{24} and RF of the formulation ME3 were significantly higher than those of the formulation ME2 and ME1, respectively ($p < 0.05$). In addition, flux, P_s, Q_s (%) and Q_{24} (%) of ME3 were significantly higher than those of ME2 and ME1, respectively (*p*

< 0.05). The penetration enhancers such as surfactants, oleic acid and isopropyl myristate in microemulsions were the key factors for penetration of these formulations. These enhancers have capability to solubilize the lipid within stratum corneum and increase drug delivery (Karande and Mitragotri, 2009). Beside the effect of surfactant and oil, skin permeation of microemulsions was a result of ethanol. Ethanol could extract skin lipids leading to increased diffusion (Karande and Mitragotri, 2009). In addition, the percent of the loading extract amount in formulations was a permeation factor. Higher percent of the loading extract amount, higher concentration gradient and driving force. The formulation ME3 had the highest ethanol concentration and loading extract amount resulting in the most improvement of skin permeation of the extract.

Table 23 Permeation parameters of the extract from the formulation ME1 and its control (n = 6, mean ± SD)

Parameters	ME1	Citrate buffer pH 5.5	*p*-value
Flux	2.820 ± 0.396	0.007 ± 0.003	0.000*
$P_s \times 10^{-4}$	0.437 ± 0.061	0.093 ± 0.037	0.000*
Q_s (%)	0.754 ± 0.040	0.260 ± 0.099	0.000*
Q_{24} (%)	0.290 ± 0.024	0.098 ± 0.021	0.000*
EF	4.678 ± 0.657	1.000	0.000*
EF of % Q_s	2.895 ± 0.154	1.000	0.000*
EF of % Q_{24}	2.943 ± 0.247	1.000	0.000*
RF $\times 10^2$	4.012 ± 0.563	1.000	0.000*

* Significant at $p < 0.05$

Table 24 Permeation parameters of the extract from the formulation ME2 and its control (n = 6, mean ± SD)

Parameters	ME2	Citrate buffer pH 5.5	*p*-value
Flux	6.081 ± 0.272	0.007 ± 0.003	0.000*
$P_s \times 10^{-4}$	1.859 ± 0.089	0.093 ± 0.037	0.000*
Q_s (%)	2.110 ± 0.261	0.260 ± 0.099	0.000*
Q_{24} (%)	1.769 ± 0.158	0.098 ± 0.021	0.000*
EF	19.886 ± 0.947	1.000	0.000*
EF of % Q_s	8.105 ± 1.001	1.000	0.000*
EF of % Q_{24}	17.966 ± 1.601	1.000	0.000*
RF $\times 10^2$	8.650 ± 0.386	1.000	0.000*

* Significant at $p < 0.05$

Table 25 Permeation parameters of the extract from the formulation ME3 and its control (n = 6, mean ± SD)

Parameters	ME3	Citrate buffer pH 5.5	*p*-value
Flux	11.953 ± 0.585	0.007 ± 0.003	0.000*
$P_s \times 10^{-4}$	2.957 ± 0.145	0.093 ± 0.037	0.000*
Q_s (%)	5.674 ± 0.156	0.260 ± 0.099	0.000*
Q_{24} (%)	3.062 ± 0.143	0.098 ± 0.021	0.000*
EF	31.636 ± 1.549	1.000	0.000*
EF of % Q_s	21.793 ± 0.598	1.000	0.000*
EF of % Q_{24}	31.099 ± 1.452	1.000	0.000*
RF $\times 10^2$	17.003 ± 0.833	1.000	0.000*

* Significant at $p < 0.05$

Table 26 Permeation parameters of the extract from the microemulsions (n = 6, mean ± SD)

Parameters	ME1	ME2	ME3	*p*-value
Flux	2.820 ± 0.396	6.081 ± 0.272	11.953 ± 0.585	0.000*
$P_s \times 10^{-4}$	0.437 ± 0.061	1.859 ± 0.089	2.957 ± 0.145	0.000*
Q_s (%)	0.754 ± 0.040	2.110 ± 0.261	5.674 ± 0.156	0.000*
Q_{24} (%)	0.290 ± 0.024	1.769 ± 0.158	3.062 ± 0.143	0.000*
EF	4.678 ± 0.657	19.886 ± 0.947	31.636 ± 1.549	0.000*
EF of % Q_s	2.895 ± 0.154	8.105 ± 1.001	21.793 ± 0.598	0.000*
EF of % Q_{24}	2.943 ± 0.247	17.966 ± 1.601	31.099 ± 1.452	0.000*
RF $\times 10^2$	4.012 ± 0.563	8.650 ± 0.386	17.003 ± 0.833	0.000*

* Significant at $p < 0.05$

For nanoemulsions, both formulations showed significantly higher all permeation parameters than those of citrate buffer pH 5.5 ($p < 0.05$) (Tables 27 and 28). So, the nanoemulsion systems could improve skin delivery of the extract. Similar to microemulsions, the effect of nanoemulsion compositions was attributed to these results.

In comparison between nanoemulsion formulations (Table 29), all permeation parameters of the formulation NE1 was significantly higher than those of NE3 ($p < 0.05$). The result indicated the better effectiveness of the formulation NE1 over NE3, which may be due to the different compositions of nanoemulsions. Surfactants could solubilize the skin and facilitate skin permeation. The effect of surfactant was consistent with the previous study of Akhtar et al. (2001) which reported that Tween® 20 provided higher flux and permeability coefficient than Tween® 80 for delivery of L-ascorbic acid in hairless rabbit skin. Moreover, the type of oil may also affect the skin permeation (Wang, Wang, and Kuo, 2007). Caprylic/capric triglyceride and soybean oil used in NE1 and NE3, respectively, could provide different skin penetration effect.

Table 27 Permeation parameters of the extract from the formulation NE1 and its control (n = 6, mean ± SD)

Parameters	NE1	Citrate buffer pH 5.5	*p*-value
Flux	8.691 ± 0.916	0.007 ± 0.003	0.000*
$P_s \times 10^{-4}$	4.093 ± 0.482	0.093 ± 0.037	0.000*
Q_s (%)	9.771 ± 0.835	0.260 ± 0.099	0.000*
Q_{24} (%)	4.046 ± 0.468	0.098 ± 0.021	0.000*
EF	43.786 ± 5.152	1.000	0.000*
EF of % Q_s	37.524 ± 3.206	1.000	0.000*
EF of % Q_{24}	41.096 ± 4.755	1.000	0.000*
RF $\times 10^2$	12.362 ± 1.304	1.000	0.000*

* Significant at $p < 0.05$

Table 28 Permeation parameters of the extract from the formulation NE3 and its control (n = 6, mean ± SD)

Parameters	NE3	Citrate buffer pH 5.5	*p*-value
Flux	5.516 ± 0.623	0.007 ± 0.003	0.000*
$P_s \times 10^{-4}$	2.488 ± 0.281	0.093 ± 0.037	0.000*
Q_s (%)	2.563 ± 0.066	0.260 ± 0.099	0.000*
Q_{24} (%)	1.564 ± 0.046	0.098 ± 0.021	0.000*
EF	26.616 ± 3.006	1.000	0.000*
EF of % Q_s	9.844 ± 0.253	1.000	0.000*
EF of % Q_{24}	15.879 ± 0.470	1.000	0.000*
RF $\times 10^2$	7.846 ± 0.886	1.000	0.000*

* Significant at $p < 0.05$

Table 29 Permeation parameters of the extract from the nanoemulsions (n = 6, mean ± SD)

Parameters	NE1	NE3	*p*-value
Flux	8.691 ± 0.916	5.516 ± 0.623	0.000*
$P_s \times 10^{-4}$	4.093 ± 0.482	2.488 ± 0.281	0.000*
Q_s (%)	9.771 ± 0.835	2.563 ± 0.066	0.000*
Q_{24}(%)	4.046 ± 0.468	1.564 ± 0.046	0.000*
EF	43.786 ± 5.152	26.616 ± 3.006	0.000*
EF of % Q_s	37.524 ± 3.206	9.844 ± 0.253	0.000*
EF of % Q_{24}	41.096 ± 4.755	15.879 ± 0.470	0.000*
RF $\times 10^2$	12.362 ± 1.304	7.846 ± 0.886	0.000*

* Significant at $p < 0.05$

To compare the skin permeation between nanosystems, the elastic liposomes, the formulations ME3 and NE1 were used as representatives for vesicular systems, microemulsions and nanoemulsions, respectively. These formulations were chosen because they showed the highest in all permeation parameters within their systems. Their permeation parameters are shown in Table 30. It was found that all permeation parameters of formulation NE1 were significantly higher than those of ME3 and elastic liposomes, respectively. This could imply that the nanoemulsions were more effective in skin delivery of *P.emblica* extract than the microemulsions and the vesicular systems. Penetration enhancers in nanoemulsions could be a reason for better skin permeation. The result was in contrast to Tavano et al. (2011) which reported that vesicular systems (niosomes) could better promote the transdermal delivery of Capsaicin than microemulsions. This may be because of difference in properties of the actives and compositions of the formulations. As seen from EF of % Q_s and EF of % Q_{24}, nanoemulsions was the best system to enhance the accumulation of the extract in skin and the permeation of the extract through skin. Thus, it was suggested that nanoemulsions was the most suitable for both dermal and transdermal delivery of the extract.

Table 30 Permeation parameters of the extract from the nanosystems (n = 6, mean ± SD)

Parameters	Elastic liposomes	ME3	NE1	*p*-value
EF	5.881 ± 0.509	31.636 ± 1.549	43.786 ± 5.152	0.000*
EF of % Q_s	6.045 ± 0.298	21.793 ± 0.598	37.524 ± 3.206	0.000*
EF of % Q_{24}	5.464 ± 0.250	31.099 ± 1.452	41.096 ± 4.755	0.000*
RF	5.876 ± 0.508	17.003 ± 0.833[(a)]	12.362 ± 1.304[(a)]	0.000*

[(a)] RF × 10^2

* Significant at $p < 0.05$

To compare concentration of the permeated extract with biological activities of the extract, the cumulative concentration of the permeated extract at 24 hr was calculated by dividing Q_{24} by volume of the receptor medium. As shown in Table 31, the cumulative concentration of the permeated extract and accumulated extract amount in skin of all nanosystems were found to be significantly higher than those of their controls. The microemulsions and nanoemulsions could deliver more extract amount to skin than that from the vesicular formulations. This was due to higher the loading amount of the extract in microemulsions and nanoemulsions. Considering IC_{50} of DPPH inhibition activity (0.878 ± 0.006 μg/mL), all nanosystems could exhibit higher concentration of the extract permeated through skin than its IC_{50} value. Among the vesicular systems, the elastic liposomes showed the highest concentration of extract permeated. The higher antioxidant activity could be obtained from microemulsions and nanoemulsions with 1.6 - 18 and 5.3 - 10.9 folds higher than IC_{50} of the extract, respectively. In addition, the accumulated extract amount in skin of all nanosystems was considerably higher than IC_{50} of the extract. However, the extract concentrations permeated through skin from all nanosystems were less than IC_{50} value of tyrosinase inhibition activity (0.539 ± 0.005 mg/mL). Because epidermis is the target site of skin lightening effect, the accumulated extract amount in skin was considered. The result indicated that the formulations ME3 and NE1 had the accumulated extract amount in skin close to the tyrosinase IC_{50} of the extract. Thus, the formulations ME3, NE1, and

elastic liposomes were suitable nanosystems for skin delivery of the *P. emblica* extract for antioxidant and skin lightening effects.

Table 31 Cumulative concentration of the permeated extract and accumulated extract amount in skin of nanosystems and their controls at 24 hours (n = 6, mean ± SD)

Nanosystems/Controls	Cumulative concentration of the permeated extract ($\mu g/cm^2/mL$)	Accumulated extract in skin (Q_s, $\mu g/cm^2$)
Conventional liposomes	1.181 ± 0.150	38.142 ± 5.698
Niosomes	0.902 ± 0.118	30.961 ± 2.079
Citrate buffer pH 5.5:PG (80:20)	0.164 ± 0.012	5.752 ± 1.000
Elastic liposomes	1.953 ± 0.089	48.648 ± 2.582
Citrate buffer pH 5.5:PG:ethanol (73:20:7)	0.342 ± 0.026	8.116 ± 1.061
ME1	2.753 ± 0.231	96.430 ± 5.124
ME2	8.734 ± 1.023	136.561 ± 16.913
ME3	21.994 ± 1.027	513.616 ± 14.099
Citrate buffer pH 5.5	0.012 ± 0.002	0.390 ± 0.148
NE1	13.261 ± 1.287	411.475 ± 31.348
NE3	6.456 ± 0.191	141.388 ± 3.634
Citrate buffer pH 5.5	0.012 ± 0.002	0.390 ± 0.148

CHAPTER V

CONCLUSION

Phyllanthus emblica (Ma-Kham Pom) fruit is well known extract used for antioxidant and skin lightening in cosmetics. The previous study of Ubonthip Nimmannit et al. (2009) did not success in delivery of *P. emblica* extract to skin by liposomes in volunteers. Thus, this study investigated the most active extract, improved entrapment efficiency of liposomes and introduced alternative carriers to solve the problem.

In this study, two extracts (spray-dried powder of *P. emblica* compressed juice and spray-dried powder of ethanolic extract of fresh fruit) were selected from the study of Ubonthip Nimmannit et al. (2009). Another two extracts (ethanolic extract of dried fruit and ethyl acetate extract of dried fruit) were practically extracted. Their biological activities and total phenolic content were investigated to obtain the most active extract. The selected active extract was then incorporated into conventional liposomes which were prepared by film hydration and microfluidization techniques. The liposomes were characterized and compared their entrapment efficiency to find the high entrapment efficiency method. Various nanosystems including elastic liposomes, niosomes, microemulsions and nanoemulsions were then prepared to deliver the extract into skin. Properties, stability, *in vitro* release and *in vitro* skin permeation of each nanosystem were investigated and compared. The experiment could be concluded as follows;

1. The ethanolic extract of dried *P. emblica* fruit exhibited high antioxidant activity by DPPH scavenging assay with IC_{50} = 1.221 ± 0.005 µg/mL, tyrosinase inhibition activity with IC_{50} = 0.519 ± 0.007 mg/mL, total phenolic content of 490.756 ± 0.185 mg GAE/g, and yield of 10.66 % - 28.56 %. Therefore, it was chosen for further formulation of nanosystems.

2. The “dissolvable extract” was obtained by re-dissolution process starting with crude extract at 20 %w/v in 95% ethanol and 60-minute sonication. The extract rendered significantly better ($p < 0.05$) antioxidant activity with IC_{50} = 0.878 ± 0.006

μg/mL, total phenolic content of 504.688 ± 0.419 mg GAE/g, gallic acid content of 26.630 ± 0.198 mg/g and solubility than the initial crude extract.

3. The entrapment efficiency of conventional liposomes prepared by microfluidization was higher than that prepared by film hydration method. Microfluidization was used for further preparation of elastic liposomes.

4. Entrapment efficiency of conventional liposomes, elastic liposomes and niosomes increased with time. There were biologically stable at 4 ºC (for liposomes) and at room temperature (for niosomes) for at least 2 months. The microemulsions (ME1, ME2 and ME3) were physically and biologically stable at room temperature for at least 2 months. The nanoemulsions (NE1 and NE3) were physically stable at the accelerated conditions and at room temperature. They were also biologically stable at room temperature for at least 2 months.

5. The extract in all formulations released slower than their controls. The percent extract release from the vesicular systems was comparable to that of microemulsions and nanoemulsions.

6. For *in vitro* skin permeation study, the extract permeation of all nanosystems was more than that of their controls. Nanoemulsions (NE1) and microemulsions (ME3) were more effective in skin delivery of the extract than the vesicular systems. Considering from EF, EF of % Q_s and EF of % Q_{24}, nanoemulsions (NE1) were the most suitable for both dermal and transdermal delivery of the *P. emblica* extract.

In the future study, nanoemulsions (NE1), microemulsions (ME3) and elastic liposomes should be further investigated for their *in vivo* skin irritation test, developed to cosmetic products and evaluated for their efficacy in volunteers.

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APPENDICES

APPENDIX A

Standard curves of gallic acid

Table A-1 Absorbance of gallic acid at various concentrations for determination of total phenolic content using Folin-Ciocalteau method

Concentration of gallic acid (µg/mL)	Absorbance			Mean	SD
1.2	0.0985	0.0982	0.0990	0.0986	0.0004
2.4	0.1764	0.1752	0.1789	0.1768	0.0019
7.5	0.4863	0.4881	0.4838	0.4861	0.0022
10.5	0.6682	0.6673	0.6657	0.6671	0.0013
15	0.9621	0.9704	0.9666	0.9664	0.0042

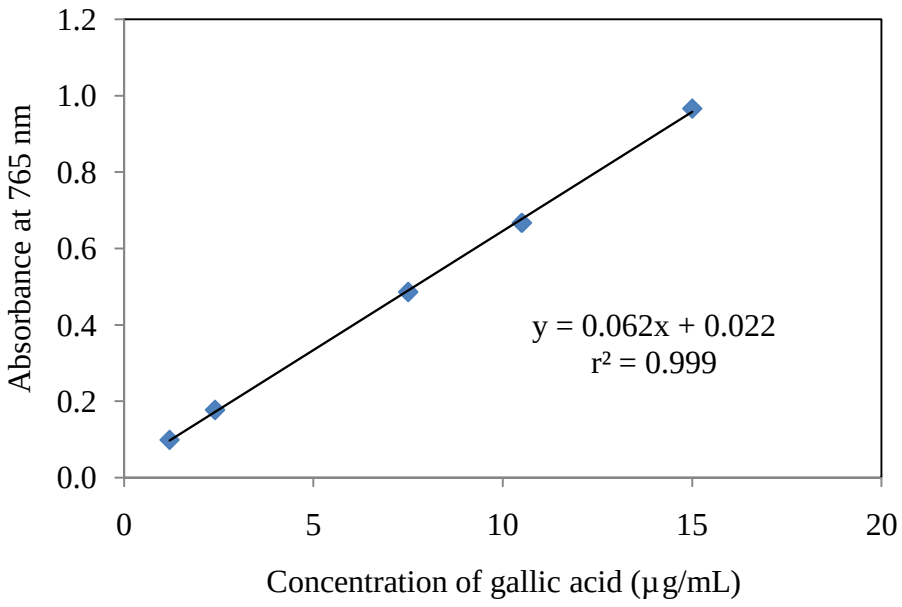

Figure A-1 Standard curve of gallic acid for determination of total phenolic content using Folin-Ciocalteau method

Table A-2 Area under curve of gallic acid at various concentrations determined using HPLC method

Concentrations of gallic acid (µg/mL)	Area under curve
10.14	283624
20.28	595591
40.56	1165819
60.84	1738124
81.12	2300653

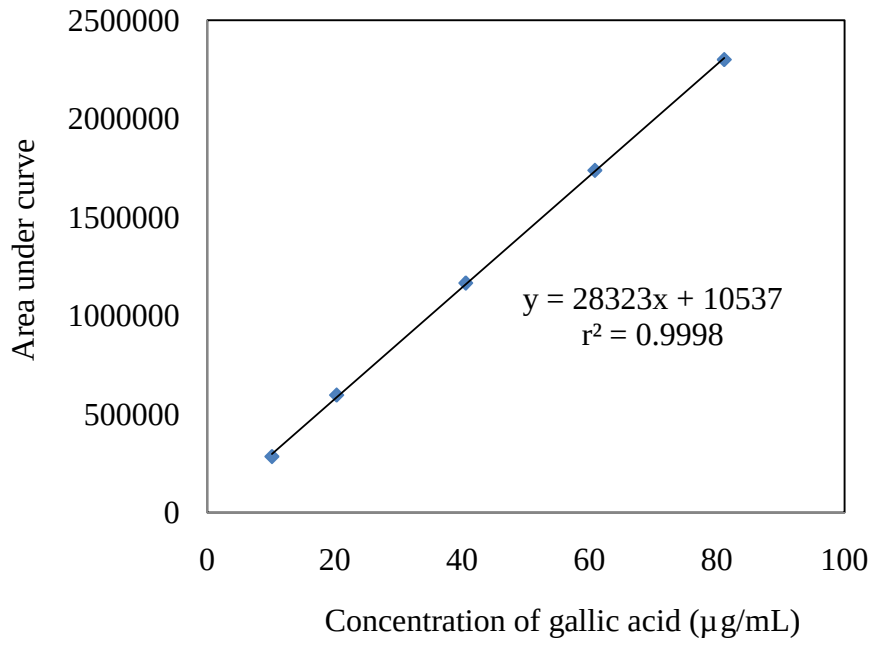

Figure A-2 Standard curve of gallic acid determined using HPLC method

APPENDIX B

The Protocol from Ethic committee

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 12-33-011	**Approval No. 12-33-011**

Protocol Title
Comparative *in vitro* evaluation of different nanosystems containing *Phyllanthus emblica* fruit extract

Principal Investigator
DUSADEE CHARNVANICH, 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	Date of Expiration
August 21, 2012	August 21, 2015

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., 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.

APPENDIX C

Validation of *P. emblica* extract assay

I. Validation of DPPH radical scavenging assay of the ethanolic *P. emblica* extract in methanol

Entrapment efficiency, extract content, *in vitro* release and skin permeation studies of the *P. emblica* extract were determined based on the DPPH radical scavenging method. The partial validation of DPPH scavenging assay was performed as the following topics (ICH Topic Q6 B, 1999; USFDA, 2001).

1.1 Specificity

The interference of the nanosystem composition on the analysis of the extract by DPPH radical scavenging assay was investigated. The analysis of the known amount of the extract in the presence of blank nanosystems was performed. Sample solutions were prepared by spiking the extract into the nanosystem composition and diluted with methanol to obtain the final extract concentrations of 2.0, 2.5, and 3.0 µg/mL corresponding to the working concentration for the extract content determination. The determination was performed in triplicate. The obtained percent DPPH inhibition was statistically compared to that of the extract in methanol at the same concentration level using Student's *t*-test. In addition, the analytical recovery (in percent) of each determination was calculated by dividing the concentration fitted from a calibration curve of the extract in methanol by the spiked concentration. The mean, standard deviation and percent coefficient of variation (% CV) were determined for each concentration. For biological assay validation, the mean value should be within ± 15 % of the actual value (Ederveen, 2010). In this study, a mean recovery in the range of 85 - 115 % was, therefore, set as the acceptable criteria.

Table C-1 shows that the percentage of DPPH inhibition of the extract solution in nanosystems was insignificantly different from that of the extract in methanol at the same concentration level except for some concentration levels of ME1 (2.0, 2.5, 3.0 µg/mL), ME3 (2.5, 3.0 µg/mL) and NE1 (2.0, 3.0 µg/mL). However, Table C-2 presents that the mean value of percent recovery in each concentration was in the range of 88.21 - 103.80 % for all nanosystems and was within the acceptable criteria. The coefficient of variation was between 0.55 - 4.23 %. The results indicated that the

studied nanosystem compositions did not affect DPPH assay of the extract in methanol. Therefore, DPPH radical scavenging assay of the *P. emblica* extract solution in methanol without nanosystems was further validated in terms of lower limit of quantitation, linearity, accuracy and precision.

Table C-1 Percent DPPH inhibition of the *P. emblica* extract in methanol and the extract solution with various nanosystems

Extraction solution with	**Extract conc. (µg/mL)**	**% DPPH inhibition**			***p*– value[(a)]**
		N1	**N2**	**N3**	
No nanosystems	2.0	60.24	59.15	58.40	-
	2.5	73.91	72.61	70.48	-
	3.0	84.79	83.40	81.31	-
Conventional liposomes	2.0	57.80	59.03	60.85	0.989
	2.5	71.32	68.91	73.54	0.818
	3.0	81.95	80.67	84.16	0.355
Elastic Liposomes	2.0	61.58	60.01	60.35	0.139
	2.5	74.32	69.60	73.00	0.713
	3.0	84.55	81.06	85.15	0.151
Niosomes	2.0	57.06	58.44	58.98	0.260
	2.5	67.73	70.34	69.85	0.120
	3.0	79.78	81.70	82.34	0.740
ME1	2.0	52.69	55.35	53.36	0.005*
	2.5	66.18	69.57	65.75	0.030*
	3.0	76.44	78.23	75.37	0.008*
ME2	2.0	58.49	57.39	56.99	0.078
	2.5	70.48	69.19	68.89	0.065
	3.0	80.74	80.44	79.99	0.055
ME3	2.0	56.26	58.57	57.46	0.098
	2.5	68.19	69.49	69.73	0.045*
	3.0	78.59	80.13	79.21	0.025*
NE1	2.0	54.87	56.10	57.03	0.017*
	2.5	68.88	69.08	70.11	0.050
	3.0	78.56	79.35	80.24	0.028*
NE3	2.0	62.60	60.05	60.79	0.112
	2.5	74.20	71.94	72.83	0.612
	3.0	85.36	82.90	83.54	0.574

(a) Statistic comparison between the extract solution with nanosystems and theextract in methanol at the same extract concentration level

* Significant at *p*-value < 0.05

Table C-2 Percent recovery of the *P. emblica* extract analyzed by DPPH radical scavenging assay and calculated using a standard curve of the extract in methanol

Extract solution with	**Extract conc. (µg/mL)**	**Fitted conc. (µg/mL)**			**% Recovery**					
		N1	**N2**	**N3**	**N1**	**N2**	**N3**	**Mean**	**SD**	**%CV**
Conventional liposomes	2.0	1.94	1.99	2.07	96.78	99.54	103.62	**99.98**	**3.44**	**3.44**
	2.5	2.54	2.43	2.64	101.71	97.38	105.68	**101.59**	**4.15**	**4.09**
	3.0	3.02	2.96	3.12	100.65	98.74	103.97	**101.12**	**2.64**	**2.61**
Elastic Liposomes	2.0	2.11	2.03	2.05	105.28	101.75	102.52	**103.18**	**1.86**	**1.80**
	2.5	2.68	2.47	2.62	107.10	98.62	104.71	**103.47**	**4.37**	**4.23**
	3.0	3.14	2.98	3.16	104.55	99.33	105.44	**103.11**	**3.30**	**3.20**
Niosomes	2.0	1.90	1.96	1.99	95.12	98.21	99.43	**97.59**	**2.22**	**2.27**
	2.5	2.38	2.50	2.48	95.26	99.94	99.06	**98.09**	**2.49**	**2.54**
	3.0	2.92	3.01	3.04	97.42	100.29	101.24	**99.65**	**1.99**	**2.00**
ME1	2.0	1.72	1.83	1.75	85.85	91.50	87.29	**88.21**	**2.93**	**3.33**
	2.5	2.29	2.43	2.27	91.59	97.34	90.85	**93.26**	**3.55**	**3.81**
	3.0	2.73	2.80	2.68	90.83	93.37	89.33	**91.18**	**2.04**	**2.24**
ME2	2.0	1.96	1.92	1.90	98.16	95.84	94.99	**96.33**	**1.64**	**1.70**
	2.5	2.47	2.42	2.40	98.89	96.69	96.19	**97.26**	**1.44**	**1.48**
	3.0	2.91	2.89	2.88	96.91	96.49	95.86	**96.42**	**0.53**	**0.55**
ME3	2.0	1.87	1.97	1.92	93.43	98.33	95.98	**95.91**	**2.45**	**2.56**
	2.5	2.38	2.43	2.44	95.00	97.21	97.61	**96.61**	**1.41**	**1.46**
	3.0	2.82	2.88	2.84	93.87	96.05	94.76	**94.89**	**1.10**	**1.15**
NE1	2.0	1.81	1.86	1.90	90.48	93.09	95.07	**92.88**	**2.30**	**2.48**
	2.5	2.40	2.41	2.46	96.17	96.50	98.26	**96.98**	**1.12**	**1.16**
	3.0	2.82	2.85	2.89	93.84	94.95	96.21	**95.00**	**1.18**	**1.25**
NE3	2.0	2.14	2.03	2.06	106.90	101.48	103.04	**103.80**	**2.79**	**2.69**
	2.5	2.63	2.53	2.57	105.20	101.37	102.87	**103.15**	**1.93**	**1.87**
	3.0	3.10	3.00	3.03	103.45	99.97	100.88	**101.43**	**1.80**	**1.78**

1.2 Lower limit of quantitation (LLOQ)

Lower limit of quantitation of the *P. emblica* extract by DPPH scavenging assay was investigated. Seven test solutions in methanol were prepared to obtain a concentration range from 0.001 to 0.50 µg/mL related to the working concentration of the extract in the permeation study. The test solution was assayed in triplicate. The percent recovery of each determination was calculated by dividing the concentration fitted from a calibration curve by the known concentration. The mean recovery, standard deviation and % CV were calculated for each concentration. In this study, a mean recovery in the range of 85 - 115 % and % CV value not exceed 15 % were set as the acceptable criteria.

From the result in Table C-3, the result of the extract concentration below 0.01 µg/mL could not be determined. The extract concentrations of 0.05 - 0.10 µg/mL showed the low percent recovery and % CV of more than 15 %. Therefore, the lower limit of quantitation of the extract in methanol by DPPH scavenging assay was 0.25 µg/mL in this study.

Table C-3Percentage of recovery of DPPH radical scavenging assay of the *P. emblica* extract with various concentrations

Extract conc. (µg/mL)	**Fitted conc. (µg/mL)**			**% Recovery**					
	N1	**N2**	**N3**	**N1**	**N2**	**N3**	**Mean**	**SD**	**%CV**
0.001	-0.55	-0.62	-0.62	N/A	N/A	N/A	**N/A**	**N/A**	**N/A**
0.005	-0.57	-0.64	-0.66	N/A	N/A	N/A	**N/A**	**N/A**	**N/A**
0.01	-0.05	-0.03	-0.03	N/A	N/A	N/A	**N/A**	**N/A**	**N/A**
0.05	0.00	0.06	0.04	-6.96	124.31	80.55	**65.97**	**66.84**	**101.33**
0.10	0.06	0.08	0.11	62.15	84.03	105.91	**84.03**	**21.88**	**26.04**
0.25	0.26	0.24	0.26	103.94	94.83	103.94	**100.90**	**5.26**	**5.22**
0.50	0.49	0.46	0.46	97.56	93.00	93.00	**94.52**	**2.63**	**2.78**

N/A: not available

1.3 Linearity

Three sets of the extract solution in methanol were prepared to obtain a concentration ranged from 0.25 to 3.5 µg/mL and were analyzed for DPPH inhibition activity. The mean of percent DPPH inhibition at each concentration was calculated. The linearity of DPPH scavenging assay of the extract in methanol was determined by plotting the standard curve between the percentage of DPPH inhibition and the concentration of extract (µg/mL). The standard curve was fitted using linear regression analysis. The coefficient of correlation (r), coefficient of determination (r^2) and the equation for the line were calculated.

The results for the evaluation of the linearity of the extract are given in Table C-4 and Figure C-1. A straight line was obtained with a coefficient of correlation (r) of 0.9998 and a coefficient of determination (r^2) of 0.9997. The regression equation of this line is

$$y = 21.863x + 14.713$$

where y is percentage of DPPH inhibition of the *P. emblica* extract in methanol and x is the concentration of extract solution in µg/mL. The result demonstrated a linear relationship between the extract concentration of 0.25 - 3.5 µg/mL in the test solution and its corresponding percentage of DPPH inhibition. The obtained calibration curve could be used for determination of the extract amount in this research.

Table C-4 Data for evaluation of the linear relationship between DPPH inhibition activity of the *P. emblica* extract and its concentration

Extract conc. (µg/mL)	**% DPPH inhibition**					
	N1	**N2**	**N3**	**Mean**	**SD**	**%CV**
0.25	20.10	19.61	20.10	**19.93**	**0.28**	**1.42**
0.5	25.00	25.49	24.51	**25.00**	**0.49**	**1.96**
1.0	36.27	37.25	38.24	**37.25**	**0.98**	**2.63**
1.5	48.04	47.55	47.55	**47.71**	**0.28**	**0.59**
2.0	58.82	57.35	59.80	**58.66**	**1.23**	**2.10**
2.5	69.61	70.10	69.12	**69.61**	**0.49**	**0.70**
3.0	80.39	79.41	81.37	**80.39**	**0.98**	**1.22**
3.5	89.71	90.69	91.67	**90.69**	**0.98**	**1.08**

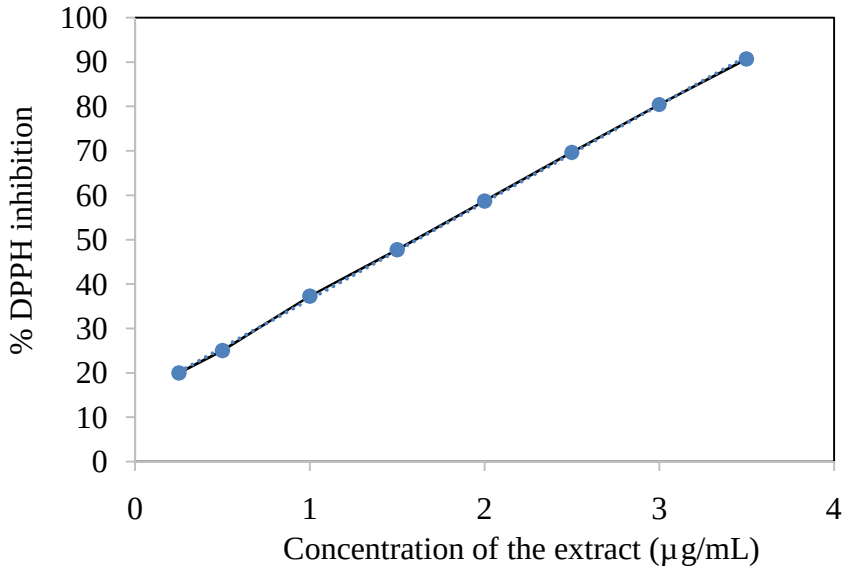

Figure C-1 Calibration curve of the *P. emblica* extract solution in methanol assayed by DPPH radical scavenging assay

1.4 Accuracy

Accuracy of DPPH scavenging assay on the analysis of the extract in methanol was performed at the extract concentration of 0.25, 1.75, and 3.45 µg/mL. Each extract concentration was analyzed in triplicate. Analytical recovery expressed in percent of each determination was calculated by dividing the concentration obtained from a calibration curve by the known concentration. The mean recovery, standard deviation and percent coefficient of variation (% CV) were determined for all determination levels. For biological assay validation, the mean value should be within 15 % of the actual value (Ederveen, 2010).

Accuracy data is presented in Table C-5. The mean value of % recovery was 98.42 % and was within 15 % of the actual value expected. The % CV value of all recoveries was 3.65 %. The 95% confidence interval ranged from 96.08 % to 100.77 %. Consequently, the theoretical value of 100 % was included. This indicated that the DPPH radical scavenging assay could be used to accurately determine content of the *P. emblica* extract within the concentration range studied.

Table C-5 Accuracy data of DPPH radical scavenging assay of the *P. emblica* extract in methanol

Extract conc. (µg/mL)	**Fitted conc. (µg/mL)**	**% Recovery**
0.25	0.22	89.75
0.25	0.25	98.72
0.25	0.25	98.72
1.75	1.75	99.92
1.75	1.70	97.36
1.75	1.79	102.48
3.45	3.47	100.71
3.45	3.38	98.11
3.45	3.45	100.06
	Mean	**98.42**
	SD	**3.59**
	% CV	**3.65**

1.5 Precision

1.5.1 Within-run precision

The within-run precision was evaluated by analyzing the extract in methanol at the concentration of 0.25, 1.75, and 3.45 µg/mL. Each concentration was performed in 5 replicates within the same day. The mean, standard deviation and % CV of each concentration were determined. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation (Ederveen, 2010).

1.5.2 Between-run precision

The between-run precision was evaluated by analyzing extract in methanol at the concentration of 0.25, 1.75, and 3.45 µg/mL. The determination was performed in triplicate. The analysis was remeasured twice in different days (between – run). The mean, standard deviation and % CV of each concentration were determined. The precision determined at each concentration level should not exceed 15 % of the coefficient of variation (Ederveen, 2010).

Tables C-6 and C-7 show data of within-run precision and between-run precision of DPPH scavenging assay of the *P. emblica* extract, respectively. The % CV values of the fitted concentrations at each concentration level of both precisions did not exceed 15 %. This confirmed an acceptable degree of precision for the determination of the extract amount by DPPH radical scavenging assay.

Table C-6 Data of within-run precision of DPPH radical scavenging assay of the *P. emblica* extract in methanol

Extract conc. (µg/mL)	**Fitted concentration (µg/mL)**							
	N1	**N2**	**N3**	**N4**	**N5**	**Mean**	**SD**	**% CV**
0.25	0.25	0.27	0.25	0.22	0.25	**0.25**	**0.02**	**6.42**
1.75	1.77	1.73	1.75	1.70	1.73	**1.74**	**0.03**	**1.47**
3.45	3.43	3.34	3.34	3.43	3.45	**3.40**	**0.05**	**1.59**

Table C-7 Data of between-run precision of DPPH radical scavenging assay of the *P. emblica* extract in methanol

Extract conc. (µg/mL)	**Fitted concentration (µg/mL)**						
	N	**Day1**	**Day2**	**Day3**	**Mean**	**SD**	**% CV**
0.25	1	0.25	0.25	0.22	**0.24**	**0.01**	**6.18**
	2	0.25	0.25	0.25			
	3	0.22	0.27	0.22			
1.75	1	1.77	1.59	1.64	**1.70**	**0.06**	**3.75**
	2	1.75	1.64	1.73			
	3	1.70	1.75	1.75			
3.45	1	3.43	3.30	3.47	**3.41**	**0.08**	**2.48**
	2	3.34	3.36	3.43			
	3	3.43	3.59	3.38			

II. Validation of tyrosinase inhibition assay of the ethanolic *P. emblica* extract in methanol

Tyrosinase inhibition assay was used to determine amount of the *P. emblica* extract for the extract content determination and biological stability of microemulsions and nanoemulsions. Tyrosinase inhibition assay of the extract in methanol was partially validated as the following topics (ICH Topic Q6 B, 1999; USFDA, 2001).

2.1 Specificity

Effect of the microemulsion and nanoemulsion compositions on tyrosinase inhibition assay of the *P. emblica* extract in methanol was investigated as the method described in topic 1.1. The extract concentrations of 0.8, 1.0, and 1.2 mg/mL related to the working concentration for the extract content determination were studied in this validation.

The result in Table C-8 shows that the percentage of tyrosinase inhibition of the extract solution with all nanosystems was not significantly different from that of the extract in methanol. Moreover, the data in Table C-9 indicated that the studied nanosystem compositions did not affect tyrosinase inhibition assay of the extract in methanol. Therefore, tyrosinase inhibition assay of the *P. emblica* extract solution in methanol without nanosystems was further validated in terms of linearity, accuracy and precision.

Table C-8 Percent tyrosinase inhibition of the *P. emblica* extract in methanol and the extract solution with various nanosystems

Extraction solution with	**Extract conc. (mg/mL)**	**% Tyrosinase inhibition**			***p*– value[(a)]**
		N1	**N2**	**N3**	
No nanosystems	0.8	62.27	61.01	60.25	-
	1.0	70.99	70.23	69.90	-
	1.2	81.19	80.53	80.04	-
ME1	0.8	62.68	63.40	61.70	0.527
	1.0	71.07	71.78	70.25	0.524
	1.2	81.21	82.03	80.44	0.961
ME2	0.8	62.18	63.43	61.15	0.271
	1.0	71.19	72.33	70.05	0.636
	1.2	81.06	82.37	79.76	0.294
ME3	0.8	62.15	61.93	61.22	0.542
	1.0	70.92	70.53	69.44	0.582
	1.2	80.83	80.17	79.74	0.571
NE1	0.8	62.27	61.24	61.51	0.921
	1.0	71.17	70.52	70.63	0.506
	1.2	80.94	79.64	80.46	0.509
NE3	0.8	62.27	61.01	60.25	0.597
	1.0	70.99	70.23	69.90	0.927
	1.2	81.19	80.53	80.04	0.678

(a) Statistic comparison between the extract solution with nanosystems and the extract in methanol at the same extract concentration level

Table C-9 Percent recovery of the *P. emblica* extract analyzed by tyrosinase inhibition assay and calculated using a standard curve of the extract in methanol

Extract solution with	**Extract conc. (mg/mL)**	**Fitted conc. (mg/mL)**			**% Recovery**					
		N1	**N2**	**N3**	**N1**	**N2**	**N3**	**Mean**	**SD**	**%CV**
ME3	0.8	0.81	0.78	0.76	100.71	97.27	95.18	**97.72**	**2.79**	**2.86**
	1.0	1.00	0.98	0.97	99.71	98.04	97.32	**98.36**	**1.23**	**1.25**
	1.2	1.22	1.21	1.20	101.74	100.54	99.64	**100.64**	**1.05**	**1.04**
ME4	0.8	0.81	0.83	0.79	101.85	103.81	99.15	**101.60**	**2.34**	**2.30**
	1.0	1.00	1.01	0.98	99.88	101.44	98.07	**99.80**	**1.68**	**1.69**
	1.2	1.22	1.24	1.20	101.77	103.27	100.37	**101.80**	**1.45**	**1.43**
ME5	0.8	0.80	0.83	0.78	100.47	103.89	97.64	**100.67**	**3.13**	**3.11**
	1.0	1.00	1.03	0.98	100.14	102.64	97.64	**100.14**	**2.50**	**2.50**
	1.2	1.22	1.25	1.19	101.51	103.89	99.13	**101.51**	**2.38**	**2.35**
NE1	0.8	0.80	0.80	0.78	100.37	99.78	97.84	**99.33**	**1.33**	**1.34**
	1.0	1.00	0.99	0.96	99.54	98.70	96.31	**98.19**	**1.67**	**1.71**
	1.2	1.21	1.20	1.19	101.08	99.88	99.09	**100.02**	**1.00**	**1.00**
NE3	0.8	0.81	0.78	0.79	100.71	97.88	98.63	**99.07**	**1.47**	**1.48**
	1.0	1.00	0.99	0.99	100.11	98.68	98.92	**99.23**	**0.77**	**0.77**
	1.2	1.22	1.19	1.20	101.29	98.91	100.40	**100.20**	**1.20**	**1.20**

2.2 Linearity

The linearity of tyrosinase inhibition assay of the extract in methanol was investigated as the method described in 1.3. The standard curve between the percentage of tyrosinase inhibition and the concentration of extract (0.2 to 1.4 mg/mL) was plotted and fitted using linear regression analysis.

The results are shown in Table C-10 and Figure C-2. A straight line was obtained with a coefficient of correlation (r) of 0.9998 and a coefficient of determination (r^2) of 0.9996. The regression equation of this line is

$$y = 45.556x + 25.564$$

where y is percentage of tyrosinase inhibition of the *P. emblica* extract in methanol and x is the concentration of extract solution in mg/mL. The result demonstrated a linear relationship between the extract concentration and its corresponding percentage of tyrosinase inhibition. This calibration curve could be used to determine the extract content in nanosystems.

Table C-10 Data for evaluation of the linear relationship between tyrosinase inhibition activity of the *P. emblica* extract and its concentration

Extract conc. (mg/mL)	**% Tyrosinase inhibition**					
	N1	**N2**	**N3**	**Mean**	**SD**	**%CV**
0.2	32.84	36.57	36.13	**35.18**	**2.04**	**5.80**
0.4	42.23	44.87	44.26	**43.79**	**1.38**	**3.15**
0.6	51.89	53.16	52.39	**52.48**	**0.64**	**1.21**
0.8	60.85	62.55	61.72	**61.71**	**0.85**	**1.38**
1.0	70.07	71.44	70.68	**70.73**	**0.69**	**0.97**
1.2	80.07	81.11	80.51	**80.56**	**0.52**	**0.65**
1.4	85.67	91.98	91.21	**89.62**	**3.45**	**3.84**

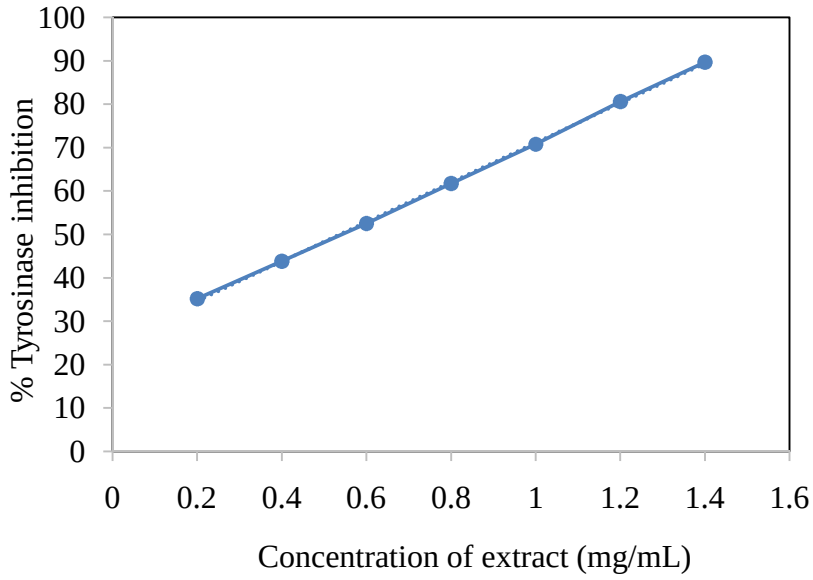

Figure C-2 Calibration curve of the *P. emblica* extract solution in methanol assayed by tyrosinase inhibition assay

2.3 Accuracy

Evaluation of accuracy of tyrosinase inhibition assay was performed as the validation method described in 1.4. The concentrations of 0.2, 0.7 and 1.3 mg/mL extract solution in methanol were used in this validation.

Accuracy data is presented in Table C-11. The mean value of % recovery was 98.83 % and was within 15 % of the actual value expected. The % CV value of all determination levels was 3.52 %. The 95% confidence interval ranged from 96.56 % to 101.10 %. Consequently, the theoretical value of 100% was included. This indicated that the tyrosinase inhibition assay could be used to accurately determine content of the *P. emblica* extract within the concentration range studied.

Table C-11 Accuracy data of tyrosinase inhibition assay of the *P. emblica* extract in methanol

Extract conc. (mg/mL)	**Fitted conc. (mg/mL)**	**% Recovery**
0.2	0.21	103.11
0.2	0.18	91.80
0.2	0.19	97.46
0.7	0.69	98.90
0.7	0.73	103.75
0.7	0.68	97.29
1.3	1.29	99.35
1.3	1.29	99.35
1.3	1.28	98.48
	Mean	**98.83**
	SD	**3.48**
	% CV	**3.52**

2.4 Precision

Within-run and between-run precisions of tyrosinase inhibition assay of the extract in methanol were evaluated as the method described in 1.5.1 and 1.5.2,

respectively. The concentrations of 0.2, 0.7 and 1.3 mg/mL extract solution in methanol were used in this validation.

Data of within-run precision and between-run precision of DPPH scavenging assay of the *P. emblica* extract are presented in Tables C-12 and C-13, respectively. The % CV values of the fitted concentrations at each concentration level of both precisions did not exceed 15 %. This confirmed an acceptable degree of precision for the determination of the extract amount by tyrosinase inhibition assay.

Table C-12 Data of within-run precision of tyrosinase inhibition assay of the *P. emblica* extract in methanol

Extract conc. (mg/mL)	**Fitted concentration (mg/mL)**							
	N1	**N2**	**N3**	**N4**	**N5**	**Mean**	**SD**	**% CV**
0.2	0.1949	0.1949	0.1836	0.1949	0.2062	**0.1949**	**0.0080**	**4.1012**
0.7	0.6810	0.6923	0.6810	0.7036	0.6923	**0.6901**	**0.0095**	**1.3706**
1.3	1.3254	1.3028	1.2802	1.2915	1.2802	**1.2960**	**0.0189**	**1.4596**

Table C-13 Data of between-run precision of tyrosinase inhibition assay of the *P. emblica* extract in methanol

Extract conc. (mg/mL)	**Fitted concentration (mg/mL)**						
	N	**Day 1**	**Day 2**	**Day 3**	**Mean**	**SD**	**% CV**
0.2	1	0.19	0.18	0.19	**0.19**	**0.01**	**4.18**
	2	0.18	0.19	0.18			
	3	0.21	0.18	0.19			
0.7	1	0.68	0.70	0.69	**0.69**	**0.01**	**1.86**
	2	0.68	0.69	0.69			
	3	0.69	0.68	0.66			
1.3	1	1.33	1.27	1.29	**1.27**	**0.03**	**2.18**
	2	1.28	1.28	1.24			
	3	1.28	1.24	1.27			

III. Effect of solvent on DPPH radical scavenging assay of the *P. emblica* extract

For *in vitro* skin permeation study, PBS pH 7.4 was used as a receptor medium. The extract in a receptor part was quantified by DPPH scavenging assay with or without dilution with methanol. A calibration curve of the extract in methanol was used for calculation of the extract amount in both cases. Effect of PBS pH 7.4 on determination of the extract by DPPH scavenging assay was investigated to confirm an acceptable degree of accuracy of this method. The extract in PBS pH 7.4 were prepared to obtain a concentration ranged from 0.5 to 3.5 µg/mL and were analyzed for DPPH inhibition activity. The analysis was performed in triplicate. The percent recovery of each determination was calculated by dividing the concentration obtained from a calibration curve of the extract in methanol by the known concentration. The mean, standard deviation and % CV were determined for each concentration. In addition, the calibration curves plotted between the % DPPH inhibition and the extract concentration were compared between those of the extract in methanol and in PBS pH 7.4. The calibration curve was fitted using linear regression analysis. Slope, intercept and correlation coefficient values of each regression line were determined and compared using the Student's *t*-test between those of the extract in methanol and in PBS pH 7.4.

Table C-14 shows that the mean recoveries of the extract in PBS pH 7.4 calculated using the standard curve of the extract in methanol were within an acceptable criteria. The same calibration curves of both the extract in methanol and in PBS pH 7.4 were obtained (Figure C-3). There were insignificant differences between slopes and intercepts of the regression lines of the extract in methanol and in PBS pH 7.4 (Table C-15). These results revealed that a standard curve of the extract in methanol could be used for the determination of the extract amount in PBS pH 7.4.

Table C-14 Analytical recovery of the *P. emblica* extract in PBS pH 7.4 assayed by DPPH radical scavenging assay and calculated using a calibration curve of the extract in methanol

Extract conc. (µg/mL)	**Fitted conc. (µg/mL)**			**% Recovery**					
	N1	**N2**	**N3**	**N1**	**N2**	**N3**	**Mean**	**SD**	**%CV**
0.5	0.38	0.48	0.42	76.80	96.87	84.83	**86.16**	**10.10**	**11.72**
1.0	0.95	0.99	0.97	94.59	98.61	96.60	**96.60**	**2.01**	**2.08**
1.5	1.55	1.49	1.43	103.20	99.18	95.17	**99.18**	**4.01**	**4.05**
2.0	2.13	2.01	2.05	106.50	100.48	102.48	**103.15**	**3.07**	**2.97**
2.5	2.57	2.57	2.51	102.86	102.86	100.45	**102.06**	**1.39**	**1.36**
3.0	3.07	2.95	3.07	102.44	98.43	102.44	**101.10**	**2.32**	**2.29**
3.5	3.43	3.47	3.45	98.13	99.27	98.70	**98.70**	**0.57**	**0.58**

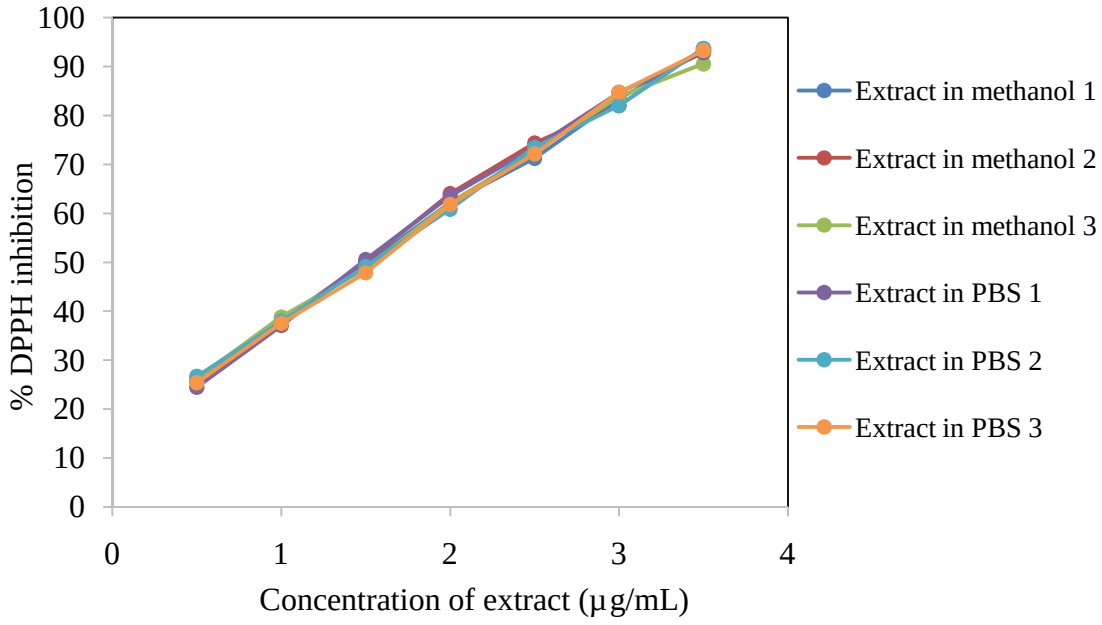

Figure C-3 Calibration curve of the *P. emblica* extract in methanol and in PBS pH 7.4 assayed by DPPH scavenging assay

Table C-15 Slope, intercept and coefficient of correlation (r) of the regression line of DPPH scavenging activity on the concentration of extract in methanol and in PBS pH 7.4

Parameters	**Extract solution in**						***p*-value[(a)]**
	Methanol			**PBS pH 7.4**			
	N1	**N2**	**N3**	**N1**	**N2**	**N3**	
Slope	23.103	22.396	23.038	22.556	22.589	21.914	0.193
Intercept	0.997	0.999	0.999	0.999	0.997	0.998	0.629
r	0.994	0.999	0.998	0.998	0.995	0.995	-

(a) Statistic comparison between the extract solution in methanol and in PBS pH 7.4

APPENDIX D

Cumulative amount of the permeated extract per diffusion area

Table D-1 Cumulative amount of the permeated extract per diffusion area of liposomes

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	1.129*	1.042*	0.762*	1.904	0.732*	0.677*	1.041	0.460
1	1.963	1.998	1.297*	2.720	1.317*	1.160*	1.743	0.598
1.5	2.528	2.689	1.950	3.584	2.059	1.782	2.432	0.663
2	3.136	3.239	2.691	4.479	2.707	2.407	3.110	0.738
3	4.292	4.601	3.847	5.782	3.857	3.570	4.325	0.802
4	5.487	5.763	4.850	7.680	4.983	4.483	5.541	1.144
6	7.818	7.336	7.463	10.509	7.048	5.945	7.686	1.523
8	9.911	8.905	9.964	13.131	9.201	7.405	9.753	1.898
12	12.366	10.240	11.848	13.919	10.757	9.142	11.378	1.693
18	14.216	12.008	14.635	15.726	12.757	11.176	13.420	1.726
24	16.018	13.293	16.872	18.102	15.141	13.325	15.458	1.931

* Below lower limit of quantification

Table D-2 Cumulative amount of the permeated extract per diffusion area of elastic liposomes

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	0.987*	1.752	2.795	1.614	0.192*	1.268*	1.435	0.867
1	2.661	3.115	3.557	2.329	1.393*	2.156	2.535	0.760
1.5	3.476	4.035	4.301	3.119	2.070	3.387	3.398	0.783
2	4.836	4.931	5.055	3.771	3.102	4.575	4.378	0.776
3	7.218	6.629	6.713	5.161	4.685	6.774	6.197	1.019
4	9.013	8.318	8.379	6.458	6.220	9.002	7.898	1.246
6	11.284	11.580	11.330	9.999	9.806	11.924	10.987	0.872
8	13.858	14.447	13.820	12.084	12.600	13.292	13.350	0.878
12	17.025	18.100	16.959	15.020	16.228	16.020	16.559	1.051
18	20.795	21.305	21.074	19.277	20.628	19.685	20.461	0.804
24	24.198	25.026	26.353	24.509	23.632	23.478	24.533	1.058

* Below lower limit of quantification

Table D-3 Cumulative amount of the permeated extract per diffusion area of niosomes

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	1.278*	0.573*	0.455*	0.061*	0.510*	0.997*	0.646	0.430
1	2.285	1.606*	1.626*	0.635*	1.170*	2.336	1.610	0.652
1.5	3.046	2.039	2.363	1.468	1.667	3.125	2.285	0.693
2	3.586	2.449	3.106	2.166	1.983	3.767	2.843	0.752
3	4.218	3.558	3.744	3.518	2.885	4.846	3.795	0.670
4	4.591	4.773	4.136	4.701	3.742	5.887	4.638	0.726
6	5.144	6.680	5.272	6.236	4.935	7.894	6.027	1.140
8	6.066	8.180	5.698	7.329	6.097	9.313	7.114	1.425
12	6.743	10.294	6.978	8.736	8.060	10.663	8.579	1.644
18	8.019	12.440	8.295	10.451	10.412	11.867	10.247	1.805
24	10.665	14.005	9.657	12.265	12.363	13.069	12.004	1.590

* Below lower limit of quantification

Table D-4 Cumulative amount of the permeated extract per diffusion area of ME1

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	0.527*	0.017*	0.317*	0.778*	0.769*	1.484*	0.649	0.500
1	0.548*	0.868*	2.072	3.145	3.189	3.499	2.220	1.271
1.5	1.953	1.862	3.643	4.895	5.145	5.310	3.802	1.580
2	3.998	3.703	5.139	6.805	7.356	7.366	5.728	1.669
3	8.320	7.480	8.220	10.556	11.036	11.443	9.509	1.695
4	12.408	11.313	11.244	14.573	15.504	16.372	13.569	2.211
6	20.553	17.749	16.883	19.488	17.853	19.765	18.715	1.422
8	24.220	19.856	21.486	21.962	20.956	22.230	21.785	1.460
12	28.998	21.120	26.310	27.291	26.556	26.712	26.165	2.654
18	34.783	27.221	30.272	31.641	31.142	32.113	31.195	2.472
24	40.968	32.043	35.477	38.145	36.709	39.062	37.067	3.107

* Below lower limit of quantification

Table D-5 Cumulative amount of the permeated extract per diffusion area of ME2

Time (hr)	Cumulative amount (µg/cm^2)						Mean	SD
0.5	0.970*	0.449*	0.829*	0.568*	1.361*	1.342	0.920	0.382
1	6.808	2.631	5.843	1.268*	6.260	6.385	4.866	2.320
1.5	9.769	4.580	9.045	3.881	9.671	8.635	7.597	2.650
2	15.099	9.652	13.233	7.912	13.559	12.948	12.067	2.708
3	22.567	16.190	19.458	16.413	20.934	20.755	19.386	2.586
4	31.690	25.587	26.759	25.510	30.463	28.746	28.126	2.596
6	48.472	41.627	43.366	40.718	47.271	43.788	44.207	3.075
8	56.526	51.790	51.830	47.950	56.260	52.671	52.838	3.205
12	71.760	69.013	66.582	63.694	74.595	68.353	69.000	3.830
18	94.238	93.983	86.228	83.500	101.611	89.806	91.561	6.489
24	118.513	117.812	106.831	103.485	131.382	108.800	114.471	10.244

* Below lower limit of quantification

Table D-6 Cumulative amount of the permeated extract per diffusion area of ME3

Time (hr)	Cumulative amount (µg/cm^2)						Mean	SD
0.5	2.290	3.518	2.472	4.130	4.554	3.062	3.338	0.901
1	4.802	4.940	5.742	6.866	6.767	6.520	5.940	0.918
1.5	9.732	8.247	8.453	11.046	8.696	9.476	9.275	1.045
2	13.547	12.482	15.290	16.168	10.318	12.022	13.305	2.165
3	32.913	33.705	31.788	33.267	30.821	30.631	32.188	1.299
4	57.545	55.869	55.061	55.692	58.347	63.071	57.598	2.952
6	83.069	83.095	84.772	85.896	84.233	92.970	85.672	3.731
8	109.511	105.044	101.915	106.769	106.711	117.844	107.966	5.443
12	155.734	145.174	141.442	146.720	146.692	160.837	149.433	7.303
18	224.721	208.510	204.279	216.629	219.150	228.338	216.938	9.239
24	287.743	265.346	259.849	275.540	280.962	293.544	277.164	12.936

* Below lower limit of quantification

Table D-7 Cumulative amount of the permeated extract per diffusion area of NE1

Time (hr)	Cumulative amount (µg/cm^2)						Mean	SD
0.5	1.315*	0.519*	0.087*	0.064*	1.212*	1.422*	0.770	0.624
1	10.978	2.503	0.860*	6.894	10.793	12.797	7.471	4.907
1.5	24.248	8.618	11.561	17.005	16.455	20.151	16.340	5.656
2	29.348	15.928	19.777	22.873	24.179	28.692	23.466	5.161
3	44.559	21.501	23.950	32.666	36.752	44.937	34.061	9.977
4	56.252	35.046	41.851	48.028	41.971	56.503	46.609	8.611
6	73.550	55.246	57.658	65.173	57.523	75.913	64.177	8.867
8	88.101	65.459	76.769	80.873	70.984	93.648	79.306	10.516
12	116.222	86.984	105.848	108.010	97.093	122.098	106.042	12.717
18	156.174	118.223	140.488	146.407	139.762	159.650	143.451	14.777
24	187.123	136.152	174.067	174.593	166.976	183.310	170.370	18.232

* Below lower limit of quantification

Table D-8 Cumulative amount of the permeated extract per diffusion area of NE3

Time (hr)	Cumulative amount (µg/cm^2)						Mean	SD
0.5	4.362	4.294	4.212	3.677	3.914	3.632	4.015	0.319
1	6.633	6.759	6.484	6.078	5.734	5.699	6.231	0.460
1.5	7.517	7.493	7.849	5.690	6.989	7.260	7.133	0.763
2	8.513	8.450	9.525	8.435	8.438	8.932	8.715	0.440
3	19.486	19.446	19.483	20.430	17.046	14.611	18.417	2.180
4	32.127	28.466	31.090	30.304	27.953	29.104	29.841	1.612
6	36.877	34.670	31.973	38.049	36.828	35.818	35.702	2.151
8	41.984	38.675	41.530	46.130	43.910	45.302	42.922	2.749
12	59.943	59.604	62.104	56.638	52.951	57.150	58.065	3.201
18	77.273	73.843	81.310	74.849	69.585	77.857	75.786	4.003
24	85.953	83.584	89.943	87.338	83.223	87.446	86.248	2.554

* Below lower limit of quantification

Table D-9 Cumulative amount of the permeated extract per diffusion area of citrate buffer pH 5.5

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	0.024	0.041	0.016	0.040	0.046	0.046	0.035	0.013
1	0.032	0.064	0.011	0.037	0.059	0.067	0.045	0.022
1.5	0.045	0.072	0.029	0.041	0.056	0.074	0.053	0.018
2	0.059	0.072	0.043	0.037	0.061	0.080	0.059	0.017
3	0.056	0.091	0.052	0.058	0.076	0.093	0.071	0.018
4	0.073	0.094	0.048	0.095	0.087	0.104	0.084	0.020
6	0.104	0.103	0.058	0.091	0.102	0.134	0.099	0.025
8	0.112	0.110	0.068	0.095	0.117	0.131	0.105	0.022
12	0.130	0.107	0.095	0.124	0.126	0.107	0.115	0.014
18	0.117	0.153	0.089	0.126	0.117	0.169	0.129	0.028
24	0.125	0.178	0.112	0.125	0.159	0.184	0.147	0.031

All data above are below lower limit of quantification.

Table D-10 Cumulative amount of the permeated extract per diffusion area of solution of citrate buffer pH 5.5:PG (80:20)

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	0.093	0.022	0.124	0.061	0.131	0.069	0.083	0.041
1	0.040	0.037	0.132	0.183	0.194	0.091	0.113	0.068
1.5	0.253	0.202	0.233	0.326	0.388	0.310	0.285	0.069
2	0.356	0.239	0.329	0.456	0.537	0.421	0.390	0.104
3	0.749	0.688	0.535	0.738	0.842	0.774	0.721	0.104
4	1.045	0.897	0.856	0.936	1.151	1.078	0.994	0.115
6	1.208	1.039	1.271	1.121	1.297	1.228	1.194	0.097
8	1.324	1.137	1.438	1.370	1.456	1.372	1.350	0.115
12	1.581	1.458	1.794	1.580	1.708	1.624	1.624	0.116
18	1.828	1.638	2.004	1.824	1.920	1.847	1.843	0.122
24	2.024	1.786	2.203	2.105	2.166	2.048	2.055	0.149

All data above are below lower limit of quantification.

Table D-11 Cumulative amount of the permeated extract per diffusion area of solution of citrate buffer pH 5.5:PG:ethanol (73:20:7)

Time (hr)	Cumulative amount ($\mu g/cm^2$)						Mean	SD
0.5	0.188*	0.060*	0.022*	0.078*	0.328*	0.310*	0.164	0.132
1	0.331*	0.165*	0.172*	0.413*	0.375*	0.273*	0.288	0.104
1.5	0.717*	0.475*	0.463*	0.556*	0.543*	0.624*	0.563	0.096
2	1.025*	0.604*	0.775*	0.778*	0.931*	0.923*	0.839	0.150
3	1.546*	0.991*	1.213*	1.348*	1.489*	1.208*	1.299	0.205
4	1.857*	1.341*	1.660*	1.859*	1.783*	1.498*	1.666	0.210
6	2.310*	1.878*	2.127*	2.475	2.544	1.878*	2.202	0.289
8	2.902	2.447	2.672	2.977	2.910	2.400*	2.718	0.251
12	3.370	2.979	3.219	3.612	3.530	3.017	3.288	0.262
18	3.909	3.617	3.671	4.275	4.153	3.521	3.858	0.307
24	4.697	4.252	4.405	4.854	4.922	4.072	4.534	0.343

* Below lower limit of quantification

VITA

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