THE DEVELOPMENT OF TRANSFERSOMES CONTAINING *EULOPHIA MACROBULBON* EXTRACT

SASAWAT POTISUWAN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Pharmaceutical Chemistry and Natural Products
2023

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By Sasawat Potisuwan

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Pharmaceutical Chemistry and Natural Products of Naresuan University

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ABSTRACT

Transferosomes were vesicular delivery systems used to carry drugs with wide range of water solubility due to amphiphilic properties. The *Eulophia macrobulbon* (EM) extract contained the active ingredient called Compound 1 having PDE-5 inhibitor. Therefore, the incorporation of the EM extract in form of transferosomes as topical formulation to improve skin permeability of EM extract was interested. The objectives of this studies were to (1) develop transdermal formulation of EM extract loaded transferosomes; (2) to investigate the skin permeability of EM extract in transferosomes and stability of formulations. The ideals of formulation were small vesicle, high entrapment efficiency, high flexibility and good stability. Transferosomes containing EM extract were prepared by film hydration method. To constructed appropriate transferosomes as drug delivery systems, the affecting parameters on the formulation such as phospholipid types [hydrogenated phosphatidylcholine (HPC, transition temperature (T_m) = 50°C) and soy phosphatidylcholine (SPC, T_m = 50°C)]; ratios of EM extract to phospholipid (1:1, 1:2 and 1:4 w/w); HLBs of edge activator (4.3-15.0); hydration mediums (pH 6.8 phosphate buffer and deionized water) were studied. The efficiency of drug entrapment, drug penetration and drug stability were quantified using high performance liquid chromatography (HPLC). Size distribution, zeta potential, deformability of transferosomes were investigated using ZetaPALS®. Drug permeability through skin was determined using Franz diffusion cell (Strat-M® as membrane). Moreover, the skin permeabilities of EM extract of transferosomes versus

solutions were compared. The larger sizes of transferosomes prepared from HPC (1897.50 ± 88.38 nm) than SPC (346.40 ± 19.40 nm) were shown. The appropriate ratio of EM extract to phospholipid was 1:2 w/w. The variation of proportions of edge activators (Span 80 and Tween 80) could provide HLBs of edge activators in the range of 4.3 to 15.0. Increasing HLB of edged activator increased the vesicle size (~350 to ~900 nm), while HLB did not affect size of transferosomes after extrusion.

The transferosome formulations composed of SPC phospholipid; EM extract : SPC ratio as 1:2 w/w; and concentration of edge activator to SPC as 15:85 % w/w with HLBs of edge activator (4.3, 9.65 and 15.0) were reduced size using extrusion method. These formulations were extruded pass through 0.4 µm pore size of membrane. The vesicle sizes of all transferosomes were comparable (approximately 200 nm). The deformability determination of transferosomes sized ~200 nm was performed using 0.1 µm pore size of membrane, then transferosomes with vesicle size ~100 nm were obtained. The deformability of these transferosomes with different HLBs (4.3, 9.65 and 15.0) of edge activator were approximately 50%. The deformability of transferosomes with increasing HLB of edge activator were similar. Increasing the concentration of edge activator slightly increased the vesicle size (779.00 ± 15.60 to 957.00 ± 45.30 nm). No effect of hydration mediums on transferosome characteristics was presented. The entrapment efficiency and zeta potential of all formulation were the same (approximately 90% and -5.0 mV, respectively). Interestingly, no effect of HLBs (4.3, 9.65 and 15.0) and transferosome sizes on skin permeability were shown in this study. Transferosomes provided better skin permeability than solutions. The influence of drug loading on skin permeability was presented. Increasing EM loading (5 and 10% w/w) in transferosomes could shorten lag time of drug permeability through the membrane, when compared with 2.5% w/w EM loading transferosomes.

The transferosomes and solutions were physically and chemically stable for 12 weeks in both 4 and 40 ˚C storage conditions. Transferosomes showed small aggregation, however it became homogenous dispersion after shaking. Size and zeta potential were not change after 12 weeks storage. No change of skin permeability of 10 weeks stored and freshly prepared transferosomes was resulted. In conclusion, EM

extract load transferosomes were successfully developed. Formulation parameters could affect the properties and skin permeability of transferosomes. The high transition temperature (T_m) of phospholipid effected the rigidity of vesicle and this probably reduced the skin permeability of transferosomes. An increase of HLB of edge activator enlarged the vesicle size due to more entrapment of other components and hydrophilic part. Transferosomes prepared from SPC provided better skin permeability compared to solutions.

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CHAPTER I

INTRODUCTION

Background and Rationale

Phosphodiesterase (PDE) is the enzyme that catalyze the hydrolysis of phosphodiesterase bond, to lesser the extent of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). (1-3) The different PDE isozymes are found in many tissues and the physiological responses are different depending on types of tissues. The inhibition of PDE can prolong physiological response mediated by of cAMP or cGMP. (1-3)

PDE-5 is the important phosphodiesterase isozyme in a role of cardiovascular system. It is mostly found in lungs and corpus cavernosum of penis. (2, 3) Inhibition of PDE-5 causes vasodilation which reduce blood pressure in lungs and increase blood flow to keep enough erection and firmness of penile for sexual intercourse.(5)

PDE-5 inhibitor is the drug used to prevent the metabolism of cGMP by PDE-5 then cGMP is accumulated, thus dilate blood vessel due to relaxation of smooth muscle. PDE-5 inhibitor is used to treat pulmonary hypertension, erectile dysfunction and also could possibly be used for Raynaud's phenomenon treatment (4). Erectile dysfunction (ED) is the inability of penile to keep erection and firm enough to have sexual intercourse or sometimes it's referred as impotence (5) and Raynaud's phenomenon is a disorder of blood vessels leading to vasospasm, discoloration of fingers and/or toes then turn white due to diminished blood flow and could turn to blue if prolonged lack of oxygen (4, 16-18). Examples of PDE-5 inhibitor drugs such as sildenafil citrate (Viagra®), vardenafil hydrochloride (Levitra®) and tadalafil (Cialis®) are used for erectile dysfunction treatment and Raynaud's phenomenon (4). Recently, oral medicament has been used for treatment of erectile dysfunction, however their costs are expensive. Moreover, oral medicament increases the risk of undesired physiological effects from systemic distribution of drug to other organs and drug interaction to other medications. For

instances of adverse drug reaction, the inhibition of PDE-1 and PDE-6 can cause of flushing and tachycardia, visual problem, respectively. (5, 8, 10, 13) These formulations are prohibited to cardiovascular disease patient because of high risk to fatal. Synergistic effect of vasodilation leading to hypotension from some drug-drug interaction could be found. (14) The other administrations of PDE-5 drug for ED treatment are commercialized. The injection of alprostadil (Carverject®) at the origin of penis shaft provide high efficacy but it causes pain after injection (15). The topical alprostadil (Vitaros®) cream for the application to glans or through intra-urethral has been formulated, however it showed less efficacy than Carverject®. By the way, those products are expensive and some of them are not distributed to every facility of health care. Therefore, a preparation of plant extraction is reasonably developed and it might be substituted and prepared as topical formulation for erectile dysfunction and Raynaud's phenomenon.

The extracts of Thai herbs, named *Eulophia macrobulbon* (EM) or Wan-Ueng (in Thai) providing PDE-5 inhibition effect have been explored. (19) EM extracts were prepared by macerated the dry power of EM tubers with 95% ethanol for 3 days, which received the high amount of extract and high quality of PDE-5 inhibitor. The active ingredient of EM extract is called as Compound 1 (Figure 1) (19). The inhibition activity of Compound 1 was compared to sildenafil as positive control. The IC_{50} of Compound 1 and Sildenafil were 1.67±0.54 µM and 0.03±0.01 µM, respectively (49). This Compound 1 possessesed PDE-5 inhibitory activity and generated vasodilation. Therefore, the EM extract might be used for the treatment of erectile dysfunction and Raynaud's phenomenon. The formulation of EM extract would be prepared in topical formulations instead of oral dosage form, to avoid possible undesired side effects of the EM extract. However, the topical formulation may provide a variety of treatment efficacy depending on the drug permeability to the target site.

From previous study, active ingredient of EM extract (Compound 1) has higher affinity to octanol than water with approximate log P as 1.61. It was soluble in methanol, ethanol, polyethylene glycol 400 (PEG 400) and propylene glycol (PG) (19), sparingly or insoluble in water. According to the properties of pure Compound 1, this active ingredient possibly passive diffused through the skin easily. However,

Compound 1 was the component of EM extract, thereby the use of whole EM extract would be used in the formulation. So, it was reasonable to prepared the suitable topical delivery system which improve the skin permeability of EM extract for erectile dysfunction and Raynaud's phenomenon treatment.

The topical delivery system was designed to deliver active pharmaceutical ingredients through the skin for local or systemic effects (51). However, the limitation of a drug to penetrate through the stratum corneum is the crucial resistance of this delivery system (23, 24). To increase the drug permeability, several methods have been introduced to weaken or temporally damaged the stratum corneum (26). The vesicular delivery systems were the methods used to overcome the permeability problem of the drug. This drug delivery system also has been reported on the improvement of drug solubility. According to the amphiphilic properties of the vesicular delivery systems, it has been used to carry drugs with a wide range of water solubility (33), therefore these systems would possibly be applied for EM extract by improving their solubility and skin permeability (32). There are several types of vesicle in these systems such as liposomes, ethosomes, niosomes and transferosomes.

Transferosomes have been categorized as elastic vesicle, due to their high deformability of vesicles to squeeze themselves pass along the intracellular sealing lipids of the stratum corneum (31, 32, 35, 36) and possibly deliver drug throughout the systemic. The important mechanism of flexible vesicular for skin penetration was osmotic gradient. This caused by the difference between water concentration of skin surface and skin interiors, which act as the driving force for elastic vesicle molecule. According to the properties of ingredients in formulation which act as skin enhancer (phospholipid and edge activator), the skin penetration pathway was not only intracellular but also intercellular and appendageal pathways. This possibly improve the skin permeability of active ingredient through the deeper layer of the skin.

There were several studies showing the better skin permeability of transferosomes than the other vesicle types of vesicular delivery systems except ethosomes. Ethosomes have higher possibility to delivery drug to deeper layer of skin because it contains high concentration of alcohol approximately 20-45 % w/w and this could be acted as skin enhancer. Unfortunately, high concentration of alcohol could irritate the skin. Transferosomes were comprised of the similar ingredients as

ethosomes but free/less alcohol. In addition, the edge activators (e.g. Span 80 or Tween 80) in transferosomes could act as skin enhancer which could avoid the irritation to alcohol. Therefore, transferosome is more suitable to prepare as topical or transdermal formulation instead of ethosomes. (34, 35)

The aim of this study is to develop a topical or transdermal formulation by vesicular delivery systems, which carry the extract for use in the treatment of Raynaud's phenomenon and erectile dysfunction. Transferosomes has been proposed to use as a carrier because its structure composed of hydrophilic and hydrophobic part which could improve the solubility of crude extract. The transferosomes would entrapped the compounds inside the vesicle and deliver through the skin. Therefore, the transferosomes is more appropriate than the conventional topical formulation (e.g. gel, cream etc.) to deliver drug through the systemic circulation. In this study, thin lipid film hydration method was used to prepared transferosomes due to the simplicity of technique and equipment in preparation,

Objectives of the study

1. To develop topical formulations of EM extract loaded transferosomes.
2. To investigate the skin permeability of EM extract with transferosomes as vesicular delivery systems
3. To determine the stability of formulations and skin permeability of formulation after storage at the temperature of 4°C and 40°C for 12 weeks.

Scope of the study

The purpose of this study were to develop a transferosomes containing EM extract as topical formulation, to study the characterization and skin permeability of transferosomes vesicle prepared by different ratios and components of formulation. The characterizations of vesicle were determined by size distribution, zeta potential and entrapment efficiency. The morphology of transferosome was determined by confocal microscope. The skin permeability of transferosomes was performed by Franz's cell diffusion apparatus with artificial skin (Strat-M®). The amount of Compound 1 from transferosomes in entrapment efficiency and the skin permeability study were analyzed by using high-performance liquid chromatography (HPLC) (19).

Keywords

PDE-5 inhibitor, *Eulophia macrobulbon*, Transferosomes, Skin permeability, Deformability.

Hypothesis of the study

Transferosomes loaded EM extract formulation could deliver active Compound 1 in the EM extract pass through the artificial skin (Strat-M®) better than solutions.

Expect output of the study

1. Topical drug formulations containing EM extract
2. The topical treatment of erectile dysfunction and Raynaud's phenomenon.

CHAPTER II

LITERATURE REVIEW

Phosphodiesterase (PDEs) and mechanism of PDE-5

Cyclic nucleotide phosphodiesterase (PDEs) were essential regulators of cyclic nucleotide-dependent signal transduction processes. They regulated 3, 5 cyclic adenosine monophosphate (cAMP) and 3, 5 cyclic guanosine monophosphate (cGMP) which are secondary messenger by hydrolysis them to their respective 5'-nucleoside monophosphates (1-2). There were several of PDEs isozyme families which located at several tissues in the body and regulated the cellular functions (1-2) and caused different physiological response of tissues and organs (2).

PDE-5 was one isozyme of phosphodiesterase found in various tissue, especially in lungs and corpus cavernosum in penis (2-3). PDE-5 had the important roles in cardiovascular system by hydrolysis cGMP and convert to inactive GMP (1-3). Inhibition of PDE-5 isozyme resulted in cGMP accumulation leading to smooth muscle relaxation (vasodilation) at corpus carvernosum. This increased blood flow then keep penile erection and also reduces blood pressure at lungs (2-3).

The PDEs form a biochemically and structurally diverse family of proteins, which has driven a search for therapeutic agents designed to inhibit specific mammalian PDEs, thereby affecting specific cellular functions (1). PDEs inhibitor drugs have been used to block the degradative action of secondary messenger such as cAMP and cGMP, leading to extent cellular functions at the specific region. Therefore, the inhibition of PDE caused the different of physiological response by the different secondary messenger where they were located (2). Sildenafil (Viagra®) was an example of PDE-5 inhibitor used to treat erectile dysfunction. There were several PDE-5 inhibitor drugs used to treat the erectile dysfunction and Raynaud's phenomenon (4).

Pathologies and treatment of erectile dysfunction disease and Raynaud's phenomenon

Erectile dysfunction (ED) was inability to get or keep erection of penis to have sexual intercourse or had short erection time (5). This problem affected an individual's quality of life (7) and could happen to every age of men but particularly old men (over 40 years old). From the previous research, prevalent of ED was 42% by the age 40-70 years old men (6) and some area would be reach at 52%.(5) Risk factors of ED were cardiovascular disease like high blood pressure, diabetics, smoke, psychotic disorders like stress or illness of mentality and patient having prostate cancer surgery etc.(5, 7) The first line of ED treatment has been oral medicaments (sildenafil citrate (Viagra®) (5, 10), vardenafil hydrochloride (Levitra®) (5, 11) and tadalafil (Cialis®). (5, 12) Some topical formulations of alprostadil (Carverject® and Vitaros®) has been available in market. The injection of Carverject® to penile caused pain and bleed (15). Vitaros® cream was administered by intra-urethral route (15). By the way, those products were expensive and some of them were not distributed to every facility of healthcare.

Raynaud's phenomenon (RP) was an artery disorder (narrowed blood vessels or vasospasm) causing less blood supplied to peripheral vessel. (4,16-18) The initial of vasospasm caused pallor following by blue and sometimes turn black and red, due to the duration of symptoms. The fingers were the most affected region but sometimes affected toes, lips, nose and ears. (4, 16-18) The affected region often happened alongside pain or tingling. (16) RP has been classified to primary and secondary stages. The primary RP occurred on its own without any associated disease typically triggered by cold or stress situation(17,18), while secondary RP occurred with associated disease, commonly linked to connective tissue disorders like an autoimmune disease such as scleroderma, Sjögren's syndrome and lupus etc. (4, 16-18) It was also resulted from blood vessels due to injury, frostbite or use of jarring machinery such as jackhammer or chainsaw (4, 17) and others factors including vasoconstrictor medication such as migraine drug etc. (4, 16-18) The treatment of primary RP was non-pharmacologic therapy by changing the lifestyle and avoiding triggered factors such as cold and stress, pharmacologic treatment would be include if non-pharmacologic was ineffective. (4, 16-18) The secondary RP treated typically

with pharmacologic with/without surgical operation depended on the stage of disease or severity of peripheral vessel damage. The injury of blood vessels led to low blood supplied at peripheral resulting in ulcer and loss of tissue of finger tips or cause gangrene. (4, 17, 18)

The treatments of secondary RP with the severity of mild to moderate have been published. First line could start by oral medication, calcium channel blockers such as nifedipine and amlodipine etc. Others treatments were PDE-5 inhibitor such as sildenafil, tadalafil and vardenafil, a topical nitrate, an angiotensin-receptor blocker (losartan) or an SSRI. (4) If the disease progressed other treatment as surgery would be required. (4, 17, 18)

From the pathology of both diseases, PDE-5 inhibitor had an important role causing vasodilation to increase blood flow at penile and peripheral. Recently, oral medication of PDE-5 inhibitor drugs for ED and RP treatments were sildenafil citrate (5, 10), vardenafil hydrochloride (5, 11), and tadalafil. (5, 12) But they might cause undesirable effect due to systemic distribution drug and their selectivity attached others isoenzymes located at different regions of the body, leading to some physiological effects. For instance, the inhibition of PDE-1 could cause of flushing and tachycardia, therefore inhibition of PDE-6 also caused visual problem. (5, 8-10, 13) The side effect of PDE-5 inhibitor can cause the fatality, especially patients with cardiovascular disease and from the drug-drug interaction between others agents like hypotensive agents. (14) The cost also expensive due to each time for treatment. The others delivery system of PDE-5 inhibitor drugs for ED treatment were available and could be used for RP. The injection formulations of alprostadil (Carverject®) have been injected at the origin of the penis shaft providing high efficacy but causing pain after administration (11). The topical creams of alprostadil (Vitaros®) hav been applied to glans or through intra-urethral, however its efficacy was less than Carverject®. (15) By the way those products were expensive and some of them were not distributed to every facilities of health care. In addition, some plants found in Thailand could provide an effect as PDE-5 inhibitor, which could possibly be used for ED and RP treatments. Then the development of topical formulation by used plant extracts these diseases was reasonable. This topical formulation would be not only cheaper but also safer than oral medication.

***Eulophia macrobulbon* (EM) possessed PDE-5 inhibition activity**

Eulophia macrobulbon (Parish, & Rchb. f.) Hook. f.in the family Orchidaceae, was investigated for PDE5 inhibitory activity (19). The active compound was 1-(4-hydroxybenzyl)-4, 8-dimethoxyphenanthrene-2, 7-diol, named as Compound 1 (**Figure 1**) (19).

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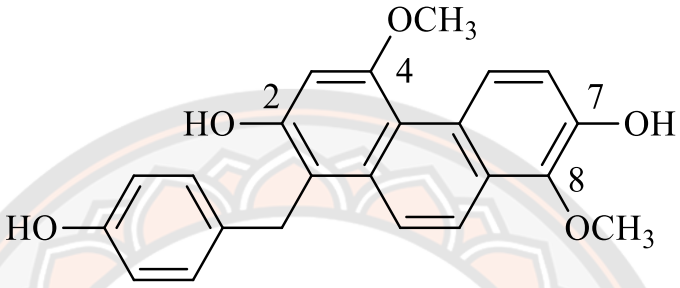


Figure 1 1-(4′-hydroxybenzyl)-4, 8-dimethoxyphenanthrene-2, 7-diol (Compound 1)

The activities of Compound 1 (IC_{50}) as PDE-5 inhibitorand positive control (sildenafil) have been studied and reported as 1.67±0.54 μM and 0.03±0.01 μM, respectively (49). From the previous study, the contraction of corpus carvernosum tissue (from reassignment surgery) for phenylephrine was dilated after the application of EM extract (0.9% w/w Compound 1) with concentration 3 mg/ml (19). This Compound 1 possessed the interesting activity in vasodilation and could possibly have the activity for ED and RP treatment.

Acute and chronic toxicity of EM extract

The acute and chronic toxicity of EM extract with oral administration were studied by following the OECD Guideline No.420 and No. 453, respectively. For acute toxicity test in rats for 2 weeks, no death, no disabilities and no disorder/damage to blood systems or internal organs from the oral administration of extract (LD_{50} level up to 2 g/kg) were found. For chronic toxicity study in rats, 3 different doses of extract (5, 50 and 500 mg/kg of body weight, once daily intake over 6 months scheduled) were conducted. The results were the same as acute toxicity test. This

implied the safety of EM extract for oral administrations. From these oral toxicity studies in rats, the EM extract could be possibly applied as topical formulation (19).

The preparation of EM extract

The EM extract was receiving from Bio-screening unit, Naresuan University. The preparation processes of EM extract included; cut *Eulophia macrobulbon* tubers into small pieces and dried in hot oven not above 60 °C. Dried materials were crushed then macerated for 3 days using 95% EtOH and filtered. The residue after the maceration was also repeated twice in the same procedure. The ethanolic filtrate was evaporated and kept at -20 °C until the studies. The physical appearances of EM extract were dark brown, sticky and viscous liquid having distinctive odor. The recent EM extract contained 0.6 % w/w of Compound 1 (19).

In the current study, the ethanolic extraction from tubers gave high amount of EM extract and high activity of PDE-5 inhibitors. The chemical components from EM extract could be separated into 11 types. The main ingredients were phenanthrene derivatives and the new compound found in this plant was Compound 1 (19) (Figure 1).

Physico-chemical properties of pure Compound 1

From the previous study (19), Compound 1 (MW = 376, Log P = 1.61 (HPLC determination)) was soluble in the different solvents; methanol, ethanol, polyethylene glycol 400 and propylene glycol. (19) The Compound 1 was chemically stable in pH 3-8 and accelerated temperature 45°C (19).

Skin structure and penetration pathways of active ingredients

Topical drug administration was interesting route due to large total surface area, approximate 1.8 m^2. (23, 24) The structure and thickness of skin varied depending on sex, age, activity and position on the body. The average thickness of skin was about 2.5 mm. The main layers of skin were divided into 3 layers as epidermis (skin protection to prevent foreign object get inside inner skin), dermis and hypodermis, which provide regulation and sensation. (23, 24)

Epidermis had a thickness about 0.1 mm. Its function was to prevent the diffusion of substances pass through and out of the skin, especially the water. The epidermis had a superficial layer called the stratum corneum (SC), which generated and peeled off every day. This layer was non-nucleated cell, and no cell division and consists of the essential cells such as keratinocyte which produced the keratin protein, the main structure of the epidermis. (23)

Stratum corneum (SC) was the major barrier for transdermal delivery system, so drug and substance could not be easily penetrated. It differed from the rest of epidermis in being a two-compartment tissue consisting of dead cornified cell (corneocytes), which keratin was the main component of cell embedded with a matrix of intercellular lipids. Corneocyte embedded in an intercellular lipid of mainly fatty acids, ceramides, cholesterol and cholesterol sulfate like brick and mortar. The corneocytes were held together by corneodesmosome, which confer to structural stability of SC. The lipids at SC such as cholesterol ceramides and fatty acids were assembled into multi-lamella bilayers. This extracellular matrix of lipid bilayers served as the primary barrier function of SC. The cell joined together by desmosome, maintaining the cohesiveness of this layer (23 - 25).

According to skin structure, there were 3 possible pathways to transport the substances via the stratum corneum by passive diffusion, which were transcellular, intercellular and appendageal pathways. In the transcellular pathway, molecules passed between the corneocyte and moved to the extracellular lipid matrix. As the lipid domain pathway, it was committed that polar molecules could difficultly permeated through transcellular pathway. According to the properties of skin on this route, the main molecules of this pathways were lipophilic (20). The lipophilic molecules with molecular weight less than 600 Da and log P 1-4 could readily diffuse through the skin (21, 22). In the intercellular pathway, molecules would cross several layers of corneocytes and extracellular lipid matrix. According to the interior of coenocytes with hydrophilic domain and extracellular matrix which were lipophilic domain, the molecules had to partition and diffuse to both domains. The intercellular pathway was often happen with small hydrophilic molecules (20). In appendageal pathway, the molecules avoided transport across stratum corneum, so they permeated the skin along the hair follicles with their associate sebaceous gland instead and

passed through sweat gland. This was the pathway for small, charge molecules and large non-charge molecules (30). At appendageal pathway the maximal delivery would correspond to 0.1-1% of applied formulation. (23 - 25)

According to the properties of Compound 1 as active ingredient of EM extract, major amount of them probably partitioned and diffused into extracellular lipid at the epidermis layer and few amount probably crossed through the deeper layers. To improve the skin permeability of Compound 1, the methods that destabilized or disturbed the structure of stratum corneum were required. This would probably increase the amount of Compound 1 across through stratum corneum with all pathways of skin and passed through the deeper layers of the skin. Compound 1 was the active component of EM extract, but EM extract would be used in the preparation in study due to the synergistic effect of all components and the expensive cost of purification. Finally, the developed formulation improving skin permeability of EM extract into the deeper layers of skin would be required.

The enhancement of skin penetration

There were several approaches to destabilized stratum corneum for improving the skin permeability of active ingredient. The enhancements of skin penetration were categorized as 4 methods. (26)

1. Chemical enhancer –damaged, disturbed the structure of SC at outer layer of skin, which caused SC lipid-fluidization and led to decreasing barrier function. Increase and optimize the thermodynamic activity of the drug in vehicle and skin. Affect the partition coefficient of drug, promoting its diffusion from the formulation to skin and disrupt the order within and between the corneocyte upon binding to the keratin filament. The examples of this group would be surfactants, organic solvent and fatty acid (27-28).

2. Change of physio-chemical properties of drug - modified the properties of active ingredient to increase solubility and permeability through the skin (26).

3. Physical enhancer – use physical approach to deliver drug via the skin portal to systemic circulation (29).

4. Vesicular delivery systems – drug entrapped by vesicle of phospholipid or single layer vesicle to deliver drug across the skin. Permeation ability of each type of

vesicle varies depending on component ratio and deformability of vesicles, such as niosomes, liposomes, ethosomes and transferosomes (31, 32).

Physical enhancer and change of physio-chemical properties of drug would not be included in this studied. Physical enhancer method required special equipment, leading to inconvenient usage. The appropriate preparation and enhancement of penetration for EM extract with convenient application was introduced.

Plant extracts were incorporated in the formulation instead of purified substances. Purification and isolation process of the constituent extract might lead to partial or total loss specific bioactivity of compounds due to loss synergy effect (33). Solubility and absorption between plant extracts and purified substance were different due to the properties of each constituent in plant extract, therefore the incorporation and dispersion methods of crude extract were required. The EM extract was probably prepared as the conventional topical formulation such as creams or solutions. Creams contained the hydrophobic and hydrophilic part, this could probably disperse the EM extract. For solutions, the co-solvent system had an important role to dissolve the EM extract. However, the safety of solvent types in the formulation were needed to be concerned. Sompod et al. (19) used the co-solvent to dissolve the EM extract. The cosolvents included ethanol, propylene glycol and deionize water (50:40:10 v/v) (19). Regrettably, the use of large amount ethanol or surfactant would cause skin irritation due to skin dryness by the loss of water. Therefore, the solutions were probably not suitable to prepared as topical formulation.

The other simply topical formulation was creams. Creams could probably be the choice for preparation. There were several studies comparing the skin permeability of vesicular delivery systems and conventional topical formulation. As the results, vesicular delivery systems possessed more advantages on the skin permeability compared to conventional topical formulations. Sanabria K et al. (50) compared the permeabilities of topical formulations of terbinafine chlorahydrate incorporated liposomes and creams. Liposomes was able to deliver active ingredient with higher amount compared to conventional cream (50). Mona Q et al. (53) did the comparison of skin permeability of miconazole nitrate incorporated with transferosomal gels and market product of miconazole nitrate cream (Daktarin® cream, 2%). The micronazole nitrate in tranferosomes had better skin permeability

than creams (53). According to the skin penetration mechanism of solution and cream, most of the active ingredient (Compound 1) of EM extract probably confined at upper layer of the skin layer due to physico-chemical properties of active ingredient which was lipophilic and main route of skin penetration of solution and cream was transcellular pathway, which also lipid domain. So, most of the active ingredient would partition in upper layer of the skin and few amount probably pass through with the deeper layers. The solution and cream were not suitable to delivered active ingredient through deeper layer of the skin.

The other comparison study of skin permeability between vesicular delivery systems and conventional topical solutions was reported. El-alim and et al. (44) compared the skin permeability of diflunisal in rat between vesicular delivery systems and solutions. Vesicular delivery system showed the better skin permeability compared to solution (44). Ghanbarzadeh and et al. (45) compared the skin permeability of diclofenac sodium in the different formulations (solutions and vesicular delivery systems) (45). Vesicular delivery systems possessed better skin permeability than solutions and followed by same as the studies of El-alim and et al. (44). These results stated vesicular delivery systems had ability to penetrate through the skin better than conventional topical formulations such as cream and solution.

According to the previously shown results of skin permeability, the vesicular delivery systems probably possessed the properties and skin penetration better than the conventional dosage forms. Therefore, the development of vesicular delivery systems had been introduced. The explanation were; (1) the carrier characteristic/structure was similar to cell membrane which improve solubility of lipophilic and hydrophilic drugs and (2) the reduction in skin irritation of drug because of drug entrapment inside the vesicle (33). There were many types of vesicular delivery systems such as liposomes ethosomes and transferosomes, based on the difference in the components characteristic and applications. (34, 35)

The comparison of vesicular delivery systems and skin penetration mechanism

The skin permeability mechanisms of vesicular delivery systems were different and this affected to skin permeability of active ingredients in the systems, based on the vesicle characteristic. The properties and skin penetration mechanisms of each vesicle type as followed:

Conventional liposomes typically composed of phospholipid such as lecithin,which was the major component of most biological membrane. The amphiphilic properties of phospholipid were widely used as drug carriers of hydrophilic (in aqueous compartments) and lipophilic drug (in lipid bilayers). The cholesterol was added into the liposomes to improve the stability and rigidity of lipid bilayers and this was reduced the skin permeability and leakage of drug payload. According to the components of liposomes, the vesicles possibly degenerated by the oxidation. To prevent the oxidation of phospholipid, the antioxidant had to be incorporated in the composition such as sodium ascorbate (58).

The skin penetration mechanisms of liposomes were proposed. The vesicles structure would be broken and absorbed on surface of stratum cornem and allowed the diffusion of active ingredient through the skin layer. The phospholipids from the vesicle would disturb the stratum corneum structure and led to formation of intercellular lipid lamella increasing the mobility of lipophilic active ingredient through stratum corneum. Furthermore, the water in vesicle increased the hydration of stratum corneum and swelling of the skin structures, this would also improve the skin permeability. All these factors would change and destabilize the stratum coreneum structure causing an increase the permeability of active ingredients. Anyways, the conventional liposomes were not suitable to prepare transdermal delivery systems. Most of vesicles stopped and ruptured at the upper epidermis layers in the intercellular pathways because of their lack of deformability, so they did not display an efficient permeation into the deeper layers of skin (59, 60). There were several studies that compared the skin permeability of nano-vesicles types. The liposomes could not deliver the active ingredient through the deeper layer on skin, so it was inappropriate to prepared liposomes loaded EM extract as topical formulation.

Niosomes were the synthetic vesicles consisting of aqueous core enclosed by non-ionic surfactant. The structure consisted of hydrophobic tails and hydrophilic heads. Based on the properties of vesicles, this resulted in the capsulation of both hydrophilic and lipophilic compounds in the vesicles. In comparison to liposomes, noisomes were chemically stable, inexpensive and no requirement of special conditions of preparation and storage. Niosome vesicles were found to be efficient in topical drug delivery as it could enhance resident time of drug in the stratum corneum as well as epidermis, and on the other hand also reduced the systemic absorption of drug.

The skin penetration mechanisms of niosomes was explained by the interaction between the vesicles and stratum corneum, resulting in fusion and adhesion to the surface. This resulted in high thermodynamic activity gradient of drug at the interaction of vesicle and surface on stratum corneum, which was the driving force for permeability of lipophilic drug cross the stratum corneum. They modified the stratum corneum structure by loosen the intercellular lipid and leading to more permeable of active ingredient. The permeation of active ingredient loaded niosomes occur via diffusion across the stratum corneum (61). Nevertheless, the niosomes were probably not the suitable systems for deliver active ingredient across through the deeper layers of skin, due to the skin penetration mechanism. There was a comparative study of the skin permeability between niosomes and others vesicle systems. Alvi et al., (62). The skin permeability of topical 5-Fluorouracil formulations in forms of noisomes, liposomes and transferosomes were compared. The transferosomes provided the highest skin permeability, due to the flexibility of vesicles and skin penetration mechanism of transferosomes (62). Gupta et al. (75) compared the skin permeability of noisome and other vesicle types using non-invasive vaccine by loaded tetanus toxoid to liposomes, niosomes and transfersomes. The vesicle sizes of all formulations were comparable but the entrapment efficiency and flexibility of transferomes was higher than others. This result indicated transferosomes permeated higher amount of tetanus toxoid compared to others. Niosomes showed lower skin permeability because they had low entrapment efficiency and and flexibility of vesicles, which could not deliver active ingredient to the deeper layer of the skin in high amount (75). From these studies, niosomes were

probably not suitable to prepared EM extract as topical formulation for erectile dysfunction disease and Raynaud's phenomenon.

Ethosomes were lipid vesicles composed of phospholipid, high content of ethanol and water. The systems were used to improve the solubility the hydrophilic and lipophilic active ingredient same as the others vesicle systems. The ethanol in the systems increased the flexibility and fluidity of vesicle, then this could improve the skin permeability of formulation. Moreover, ethanol would act as skin enhancer for vesicles, it disturbs the organization of lipids in stratum corneum, leading to an enhancement of the fluidity and a decrease in the density of intercellular lipid domains. This system could promote the penetration of active ingredient loaded in ethosomes through the deeper layers of the skin. The studies of ethosomes as effective carrier for topical application were published. Ethosomes showed significant advantages of skin permeability over other vesicle systems, because of vesicle properties and high content of ethanol. El-alim et al. (44) compared the skin permeability of diflunisal formulations in rat, using liposomes, transferosomes, ethosomes and solutions. Etosomes showed the highest skin permeability compared to other systems (44). Ghanbarzadeh et al. (45) compared the skin permeability of diclofenac sodium in the different vesicle types which were liposome, ethosomes and transferosomes [(45)]. Ethosomes provided the highest skin permeability. The same result was presented by El-alim et al. (44). However, ethosomes could cause some irritation on the skin due to the high ethanol content (20-45%) in the system (35, 36). So, ethosomes were probably not a suitably topical formulation for Erectile Dysfunction and Raynaud's phenomenon treatment. Another nano-carrier system which possessed flexibility characteristic and improved the skin permeabilitywas introduced. Transferosomes were probably used to prepared EM extract topical formulation instead of ethosomes.

Transferosomes are lipid base vesicular carriers composed of phospholipid bilayers with an additional component such as edge activator (e.g. Tween80, Span80). Edge activator was inserted between phospholipid and led to disturb the structure of bilayers. This made transferosomes having less stiffness and better skin permeability than liposomes. The amphiphilic properties of transferosomes would probably improve the solubility of active ingredients and entrapped both hydrophilic and

lipophilic ingredients in vesicle. The skin permeability mechanism of transferosomes was compared to other systems. When drug delivery systems such as liposomes, noisomes and nano-particles deposited on the skin, they were only able to penetrate through upper layers of stratum corneum, resulting in accumulation in the epidermis layer unable to reach the deeper layers of the skin such as dermis layer or systemic.

Penetration mechanisms of transferosomes were discussed. The important mechanism of skin penetration was osmotic gradient, inducing the difference of water concentration between dehydrate skin surface (~15% of water) and skin interiors (water content ~75% of water). The difference in hydration was the physiological feature of water loss. Based on the principles of elastomechanic, hydrophilic vesicles established an energetically favorable interaction, which forced them move from the rather dried environment to more hydrated regions. When the transferosomes were place on the skin which partly dehydrated by water loss, the transferosomes vesicle perceived osmotic gradient and avoided the dehydration by deformation of the vesicles and moving themselves along to more hydrated regions in the deeper layer of the skin. (34-36, 63). This was a non-occlusive condition, which was essential for transferosome penetration due to the maintenance for osmotic gradient to be the driving force of transferosomes vesicle (47, 48).

The penetration of tranferosomes was not only the intercellular pathwaybut also the permeation through skin by 3 pathways, which were intercellular, transcellular and appendageal. The vesicles deposited on stratum corneum and disturbed the skin structure, this led to increase the fluidity of the skin and allowed active ingredient to diffuse through the deeper layers of the skin such as dermis. The several skin permeabilities of transferosomes have been studied and compared.

Zhang Z. et al. (64) studied the skin penetration of 5-fluorouracil loaded in transferosomes and liposomes. The formulations were tagged by rhodamine 6GO to determine the fluorescent intensity in the skin layer. Transferosomes provided better skin permeability compared to liposomes. The study showed most of the transferosomes permeated through deeper layer but most of the liposomes deposited on stratum corneum and damaged the upper layer of the skin by the time (64). This result related to the studied of Mahmood S. et al. (65). Raloxifen loaded transferomes, liposomes and ethanolic solution were prepared. All formulations were tagged by

fluorescent 6-coumarin. The intensity of fluorescence of transferosomes in skin layers were highest compared to other systems (65). The transferosomes were suitably prepared EM extract as topical formulations for erectile dysfunction and Raynaud's phenomenon treatment, because these transferosomes could improve the solubility of active ingredient and possibly delivered active ingredient across through the stratum corneum and reached to the deeper layer of the skin such as dermis or systemic.

Transferosomes with high entrapment efficiency and small vesicle size were the goals of preparation (39). The vesicle ranged of 200-300 nm could easily penetrate through SC (40). The compositions of formulation and preparation methods would play an important role on the morphology and vesicle characterization. The influencing parameters on the vesicle size and entrapment efficiency were phosphatidylcholine concentration; phosphatidylcholine type; amount, type and HLB of surfactant; temperature of preparation. Small size transferosomes could be obtained by decreasing phosphatidylcholine concentration, using low Tm phosphatidylcholine and increasing HLB of surfactant. High entrapment efficiency of transferosomes could be achieved by increasing phosphatidylcholine concentration, surfactant concentration, preparation temperature (temperature must be above the Tm of phosphatidylcholine) and decreasing HLB of surfactant. (39, 42) Several methods for transferosome preparation have been used. Each prepared method might produce the different range of vesicle size. Classical methods of transferosomes preparation were lipid thin film hydration prepared by cast thin film or lipid cake then hydrated by medium. The size of transferosomes obtained from these methods was in range of 1 µm – 5 µm. Reverse phase evaporation would be prepared by mixing lipid components and aqueous then evaporating organic solvent, the range of vesicle sizes was <100 nm – 1 µm. Solvent injection technique was prepared by injection of lipid solution into aqueous medium to form the vesicle, the range of vesicle sizes was <100 nm. The dialysis of vesicles was needed, when lipids were solubilized by detergent. Then, mixed micelles and detergent were removed by dialysis, the range of vesicle size was 80 nm -140 nm. Among these preparation techniques, reverse phase evaporation and solvent injection technique could be enlarged for production scale (52). The different preparation methods involved different equipment and complexity,

so simple equipment and preparation method was appropriate to prepare and characterize transferosomes in this study.

Preparation and determination of transferosomes

In this study, the transferosomes were prepared by lipid thin film hydration method and followed with vesicle size reduction by extrusion method. (37) This method is simply prepared and completely evaporated the organic solvent. This method was suitable for low quantity production, good for lab scale but it was not for larger scale production. Thin lipid film was prepared by dissolving lipophilic contents (phosphatidylcholine, lipophilic drug and surfactant) in organic solvents (e.g. (ethanol-chloroform) (37, 38). Organic solvent in the process would be evaporated by evaporator with temperature above the lipid transition temperature (T_m). Final traces of solvent would be removed under vacuum overnight. The forming thin film will be hydrated with pH 6.8 phosphate buffer solution in the same evaporator with 60 rpm for 2 hrs. at the corresponding temperature. The prepared transferosome suspensions would be formed with milky appearance. The size reduction of transferosomes using the mini-extruder with polycarbonate membrane was employed. Homogeneity of transferosomes was controlled by manual extrusion at least 10 times through membranes pores sized 400 or/and 100 nm, respectively (37, 38).

The structure of transferosomes containing EM extract would be characterized.Vesicle size and zeta potential were determined using Malvern Zetasizer. Vesicle morphology was investigated using photon correlation or dynamic light scattering (34, 37, 38). Transferosomes could be visualized using phase contrast microscopy or confocal microscope. The stability of transferosomes could be determined through the vesicle size and structure changing over time by dynamic light scattering. The quantitative determination of Compound 1 in the prepared transferosomes would be determined using HPLC. The entrapment efficiency was determined by centrifugation method. Accurate weighted compounds were loaded and dispersed in DI water and centrifuged by speed of rotation approximately 15,000 rpm. The pellets and supernatant were determined using HPLC assays and the percentage of entrapment was calculated (34, 38). Deformability of transferosomes was performed by extrusion method. The formulations were passed through the

polycarbonate membrane with pore size 100-200 nm and the differential of vesicle size was calculated and determined as the percentage of deformability.

In-vitro skin permeation and artificial membrane (Strat-M®)

The in-vitro skin permeation studies was determined using Franz's diffusion cell (34, 37, 38) with 7.0 ml receiver compartment, effective diffusion area of 1.77 cm2 (37, 38) and synthetic membrane were used. Strat-M® was a synthetic membrane that use in several studies for skin permeability test, due to its similarity of structural and chemical properties as human skin. The Strat-M® membrane was constructed of many layers and thickness of each layers approximately 300 µm. The tight top layer was supported by 2 layers of porous polyethersulfone (PES) on top of one single layer of polyolefin non-woven fabric support. There are several porous in each layers of membrane. The porous increased the thickness of the Strat-M® membrane to mimic the layers of human skin (epidermis, dermis and subcutaneous).

The porosities in membrane were impregnated with blend of synthetic lipid such as ceramides, cholesterol, free fatty acid and others components, which impart hydrophobicity of skin characteristic. The synthetic lipids in Strat-M® mimicked to the impregnated lipids in human stratum corneum in specific ratio and lipid types. The efficacy of Strat-M® has already been investigated and compared to human cadaver skin by Anika Hand et al. (43). The experiment was performed using nicotine solution to determine and compare the skin permeability of each type of the skin. Each solution contained the different types of skin enhancers including Azone, Eucalyptol, N-methyl Pyrolidone and Tween 80. The skin permeability results of nicotine solutions on both skin types showed that the Strat-M® membrane demonstrated the high relative correlation to human cadaver skin with R^2 of each formulation as 0.90, 0.98, 0.99 and 0.99, respectively. This could confirm the similarity between Strat-M® and human skin. The integrity or stability of skin was important for long time period of the diffusion study of extended-release formulation. The human/animal skin deteriorates in 20-24 hrs. under in-vitro release experiment. This became a limitation of human skin usage to perform extend in-vitro release by using human/animal skin model. Strat-M® provided more stability of membrane, then it could be easily used for long term diffusion study. However, metabolized active

ingredients by membrane would not happen to Strat-M® as occurred in human/animal skin. The good correlation of Strat-M® and human/animal skin have been validated, even though the application of Strat-M® as the replacement for human/animal skin was needed more studies to confirm. Strat-M® had long shelf life and more convenience compared to human/animal skin, this was reasonable for using Strat-M® instead of human skin for preliminary screening the skin permeability. While the animal skin was involved the several preparing process prior to use.

The receptor compartment was filled with pH 7.4 phosphate buffer saline maintained at 32.0 ± 1.0˚C and stirred by a magnetic bar with the speed 300 rpm. According to OCED guideline the receptor fluid had to be sink condition and not limited the permeability of drug. For lipophilic drug, the receptor fluid might be contained mixture solvents of ethanol (not exceed 50% aqueous ethanol), <6% polyoxyethelene oleyl ether in water or 5% bovine serum (41). Ethanol in the solvent was necessary due to the lipophilic properties of Compound 1 (43).

CHAPTER III

RESEARCH METHODOLOGY

Materials

Ethanolic *Eulophia macrobulbon* (EM) extract (Naresuan University, Phitsanulok, Thailand); soy phosphatidylcholine (SPC) (Lipoid, Ludwigshafen, Germany) ; hydrogenated phosphatidylcholine (HPC) (Lipoid, Ludwigshafen, Germany) ; Span® 80 (hydrophilic-lipophilic balance (HLB) = 4. 3) (NOF CORPORATION, Tokyo, Japan); Tween® 80 (hydrophilic-lipophilic balance (HLB) = 15.0) (NOF CORPORATION, Tokyo, Japan); Carbopol® Ultrez 21 (Lubrizol, Ohio, United States); potassium dihydrogen phosphate (Ajax Finechem, Australia); sodium hydroxide (Ajax Finechem, Australia), absolute ethanol (RCI Lab scan, Bangkok, Thailand), acetonitrile (ACN) (RCI Lab scan, Bangkok, Thailand); deionized water (RCI Lab scan, Bangkok, Thailand); GermaBen™ (Ashland, USA) and propylene glycol (PG) (REPSOL, Madrid, Spain) were used as received.

Methods

1. Validation of analytical method for determination of Compound 1 in EM extract

EM extraction

EM extract was obtained from Bio-screening unit, Naresuan University. Briefly, the identified tubers of EM were purchased from Prachinburi. The tubers were then extracted according to the patent application No. 1503001282. The EM extract was brown and viscous resinous extract. It contained approximate 0.6% w/w of Compound 1. The extract was not completely dissolved in both hydrophilic and hydrophobic solvent (19).

Quantitative determination of Compound 1

The amount of Compound 1 was determined using HPLC-UV assay which firstly studied by Sompod et al. (19). The determination was performed using SHIMADZU LC solution (Shimadzu Corporation, Japan) equipped with a solvent delivery pump (LC-20AT), UV/Vis as a detector (SPD- 20A) and an auto-sampler

(SIL-20A) with a 20 µL loop. The separation was performed with a reversed phase column Gemini®-NX 5 µm C18 110Å 150x4.60 mm i.d., 5 µm particle size (Phenomenex, CA). The gradient mobile phase with the flow rate of 1 ml/min was programmed as following: 0.0-5.0 min, linear gradient 38% ACN; 12.5-13.5 min, linear gradient 100% ACN; and final washing up of the column with gradient 38% ACN for 6 min before reconditioning the column. Compound 1 was detected at the wavelength of 265 nm. Data was collected and manipulated using CLASS-VP™ System Software (Shimadzu Corporation, Japan). The standard curve of pure Compound 1 was created by plotting the AUC of the peak of pure Compound 1 versus the concentration in the range of 0.1-5 µg/ml. The linearity was obtained by linear regression analysis calculated by the least square regression. The intra-day, inter-day precision and accuracy was conducted at the concentration of 0.2, 0.6 and 3 µg/ml (n=3) and presented as % RSD (precision) and %recovery (accuracy) (19).

$$\%RSD = \frac{SD}{average} \times 100$$

$$\%accuracy = \frac{determined\ concentration}{theoretical\ concentration} \times 100$$

2. Preparation of EM extract loaded transferosomes and solutions

2.1 Tranferosomes (2.5%, 5.0% and 10.0% w/w of EM extract)

Transferosomes containing 2.5 and 5.0% w/w of the EM extract were prepared by lipid film hydration technique (40, 37). The ingredients with different ratios (**Table 1**) were mixed then dissolved in absolute ethanol, sonicated until solution obtained (sonicator, Darwell Ultrasonic Cleaner, with work frequency 40 kHz and power 90%). The used organic solvent was removed using rotary evaporator (Buchi Rotavapor® R-300) (90 rpm under 105 mbar pressure, 1 hr), then hydrated the lipid film with a medium (pH 6.8 phosphate buffer USP) for 2 hr. Transferosome sizes was decreased using a mini-extruder (52) (Avanti Polar Lipid Inc., Alabaster, Alabama, USA) equipped with polycarbonate membrane (0.4 and 0.1 µm pore size). The temperatures above the transition temperatures (T_m) of phospholipids (40 °C for soy phosphatidylcholine and 60°C for hydrogenated phosphatidylcholine) were

maintained during the size reduction. Final products were determined as following experiment (section 3.2.3). The optimized ingredients and ratios (**Table 1**) of the formulation were chosen to prepare EM extract loading with 5.0 and 10.0 % w/w. Formulations with 10.0% w/w were prepared by reverse-phase evaporation method (34, 38). The ingredients with optimum ratios were mixed then dissolved in absolute ethanol, sonicated until solution obtained (sonicator, Darwell Ultrasonic Cleaner, with work frequency 40 kHz and power 90%) then added PBS and sonicated for 30 minutes. The organic solvent was removed using evaporator, rotated with 90 rpm and pressure 1 05 mbar at 40 °C until the total weight of formulation constant then adjusted the weight with PBS in case total weight of formulation was less than required amount.

2.2 Solutions

The EM extract solutions were prepared by incorporation 2.5% (w/w) of EM extract and 5% (w/w) Transcutol® (skin enhancer), then dissolved and adjusted volume by co-solvent which composed of ethanol, propylene glycol and deionized water (50:40:10 v/v) (19).

Table 1 The parameters used for EM extract loaded transferosomes preparation and studied vesicle characterization (size, zeta potential and entrapment efficiency)

Parameters			
Phospholipid type (T_m, °C)	SPC (-20°C)	HPC (50 °C)	
Crude extract : PC (w/w)	1:1	1:2	1:4
Surfactant : PC (%, w/w)	10:90	15:85	20:80
HLB of surfactant	4.3	9.65	15.0
Hydration medium	PBS, pH 6.8	Deionized water	

Table 2 The transferosomes formulation used to prepared and studied vesicle characterization (size, zeta potential and entrapment efficiency)

Formulation code	Crude loading (%, w/w)	Crude : Lipid ratio (w/w)	HLB of edge activators	Lipid ratio : Edge activator (%, w/w)	Hydration medium
SPC-$1_{(Sp80)}$	2.5	1:2	4.3	85:15	pH 6.8, PBS
HPC $_{(Sp80)}$	2.5	1:2	4.3	85:15	pH 6.8, PBS
SPC-$2_{(Sp80)}$	2.5	1:1	4.3	85:15	pH 6.8, PBS
SPC-$3_{(Sp80)}$	2.5	1:4	4.3	85:15	pH 6.8, PBS
SPC-$4_{(Sp80+Tw80)}$	2.5	1:2	6.9	85:15	pH 6.8, PBS
SPC-$5_{(Sp80+Tw80)}$	2.5	1:2	9.6	85:15	pH 6.8, PBS
SPC-$6_{(Sp80+Tw80)}$	2.5	1:2	12.3	85:15	pH 6.8, PBS
SPC-$7_{(Tw80)}$	2.5	1:2	15.0	85:15	pH 6.8, PBS
SPC-$8_{(Tw80)}$	2.5	1:2	15.0	95:5	pH 6.8, PBS
SPC-$9_{(Tw80)}$	2.5	1:2	15.0	90:10	pH 6.8, PBS
SPC-$10_{(Tw80)}$	2.5	1:2	15.0	80:20	pH 6.8, PBS
SPC-$11_{(Sp80)}$	2.5	1:2	4.3	85:15	DI water
SPC-$12_{(Tw80)}$	5.0	1:2	15.0	85:15	pH 6.8, PBS
SPC-$13_{(Tw80)}$	10.0	1:2	15.0	85:15	pH 6.8, PBS

SPC, HPC, Sp80 and Tw80 mean soy phosphatidylcholine (T_m - 20°C) and hydrogenated phosphatidylcholine (T_m 50°C), Span® 80 and Tween® 80 respectively. The mixing of Sp80 and Tw80 produced different HLB of edge activator. The total amount of each formulation was 10.0 g.

3. Characterizations of transfersomes containing EM extract

3.1 Morphology

One hundred microliters of transferosomes were diluted with hydration medium to 1.0 ml and dispersed. Morphology of transferosomes was observed by using a confocal laser scanning microscope (Nikon A1Rsi, Nikon Instruments Inc., New York, USA).

3.2 Vesicle size, size distribution and zeta potential

Vesicle size, polydispersity index (PDI) and zeta potential of transferosomes were characterized using dynamic light scattering (DLS; ZataPALS®, Brookhaven Instrument Corporation, Holtsville, USA). The instrument was equipped with a 35 mW Helium-Neon laser diode operating at 632.8 nm and BI-200SM Goniometer connected to BI-9010AT digital correlator. The vesicle size and PDI were obtained by the auto measuring mode at a fixed angle of 90° (38). Twenty microliters transferosomes were diluted and dispersed with hydration medium to 3 ml of the cuvette. The determinations of vesicle size and zeta potential with 6 repetitions cycles at 25 °C,. The zeta potential with an angle of 14.8° to the incident light was performed (38).

3.3 Determination of entrapment efficiency (EE)

One hundred milligrams of transferosome suspensions were adjusted with 1 ml hydration medium and fully dispersed in the Eppendorf tube. The entrapped transferosomes were separated using ultra-centrifugation (Hettich® MIKRO 220R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) with 15,000 rpm at 4 °C for 30 minutes (34).

Transferosome vesicles with the approximate size 90-150 nm were used. The entrapment efficiency was determined by dispersed the formulation in Eppendorf tube (500 µl total) with filter (100 kD pore size) and centrifuged by 12,000 rpm at 4 °C for 30 min. Separated the transferosome vesicles and supernatant then

dissolved each part with methanol. Determined the amount of Compound 1 of each part by HPLC assay and calculated with the equation.

$$\textbf{Compound 1 entrapment (\%)} = \frac{\textbf{entrapped in pelltes}}{\textbf{entrapped in pellets+supernatant}} \textbf{ x 100}$$

3.4 Deformability measurement

One milliliter of transferosomes was added in the device and passed through a mini-extruder (Avanti Polar Lipid Inc., Alabaster, Alabama, USA) with 100 nm pore size of polycarbonate membrane at 32±1 °C. The vesicle sizes were measured and calculated deformability by the followed equation (38).

$$\textbf{Deformability (\%)} = \frac{\textbf{Vesicle size before extrusion} - \textbf{Vesicle size after extrusion}}{\textbf{Vesicle size before extrusion}} \textbf{ X 100}$$

4. In- vitro permeation studies

The in vitro skin permeation using vertical Franz diffusion cell (Teledyne Hanson Research, California, USA) was studied (19, 38). The study condition (1.77 cm^2 diffusion area; 7 ml receptor chamber; reservoir solution (pH 7.4 phosphate buffer, ethanol and propylene glycol, 10:50:40 v/v) (41); temperature 32±1 °C; magnetic bar speed 300 rpm; Strat-M® (Merck KGaA, Darmstadt, Germany) as artificial skin) was conducted. Weighted 0.3 g of samples and placed over Strat-M®. At a predetermined time, one-milliliter reservoir solution was withdrawn and replaced with a same amount of pre-warm fresh medium. After the study, the left formulation on the membrane, Strat-M® membrane and reservoir medium was extracted and quantified by HPLC assay. The solubility of Compound 1 in EM extract in this receptor medium was 669 ±35 µg/ml. This study was performed under sink condition (0.6% w/w of Compound 1 in EM extract, concentration of EM extract ~116.5 mg/ml) (19). The transferosomes with 2.5, 5.0 and 10.0 % w/w EM extract loading in the 0.3 ml of transferosomes would provide approximately 7.5, 15.0 and 30.0 mg of extract or 45, 90 and 180 µg of Compound 1, respectively.

5. Stability test

The formulations in tight container were stored at temperatures 4-8 °C (refrigerator) and 40 °C (oven) (46). Vesicle size, size distribution, zeta potential and amount of Compound 1 in the formulations at 1, 2, 4, 8 and 12 weeks were investigated. The stabilities of Compound 1 and skin permeability of both conditions were determined.

CHAPTER IV

RESULTS AND DISCUSSION

Determination of Compound 1

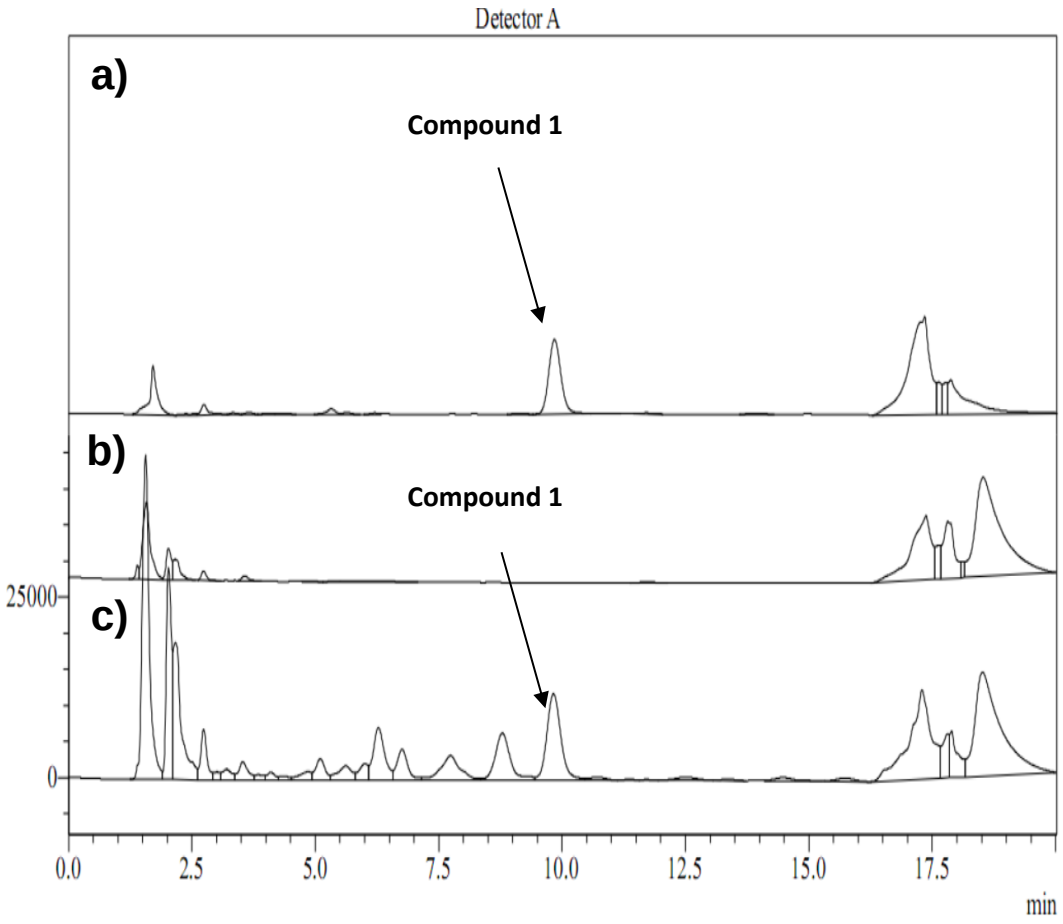


Figure 2 HPLC Chromatogram of STD Compound 1 a concentration of 0.5 µg/ml (a), receptor fluid (EtOH: PG: water) and blank transferosome (b) and EM extract loaded transferosomes in receptor fluid (c)

Compound 1 was a marker of EM extract. The Compound 1 was determined and quantified by using the HPLC method, which was developed by Sompod et al. as previously described in the methodology (19). The entire determination was carried out over a period of 20 minutes. The retention time of Compound 1 was 9.8 min. The chromatogram of the pure Compound 1, a blank penetrating receptor fluid and the EM extract loaded transferosomes was compared. The results indicated the specificity of the method to the compound 1 due to the un-overlapping of the other peaks (Figure 2). The standard curve of compound 1 was constructed and exhibited a linear correlation in the range of 0.1-5 µg/ml with the R^2 of 0.9995 (Figure 3). The intra-day and inter-day precision (%RSD) were in the range of 3.74-5.63 and 2.88-5.77%, respectively. The accuracy (% recovery) was in the range of 94.39-102.94% with the %RSD of 1.08-5.96%. Both precision and accuracy were within an acceptable range according to the AOAC guideline (precision not more than 16%RSD, accuracy in the range of 75-120%recovery). Corresponding to Sompod et al., the limit of detection and the limit of quantitation of this HPLC method was 1.25 and 5 ng/ml, respectively (19).

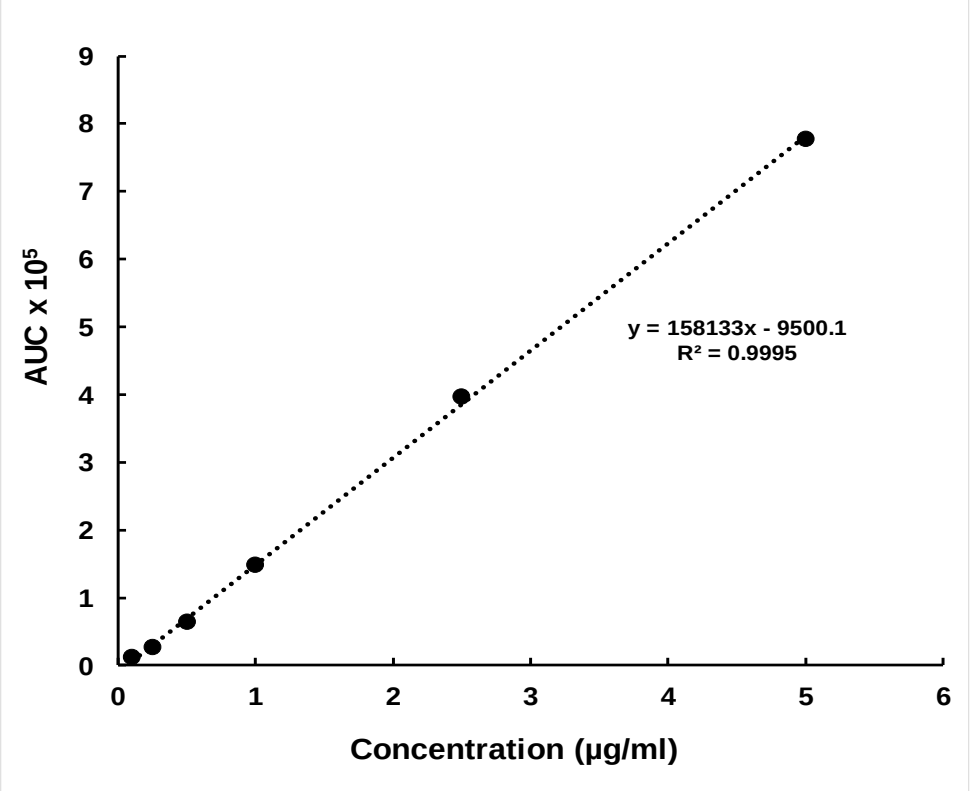


Figure 3 Standard curve of Compound 1, consist of six standard points as 0.1, 0.25, 0.5, 1.0, 2.5 and 5 µg/ml. The correlation coefficient (R^2) was 0.9995

Preparation of transferosomes

The transferosomes with 2.5 and 5.0 % w/w EM extract was prepared by lipid thin film method. The vesicle size reduction of transferosomes by extrusion method was performed. In addition to 10% w/w of EM extract loading, transferosomes were prepared by reverse-phase hydration method. This preparing method for 10% w/w of EM extract was used because the hydration process of lipid thin film method had not enough liquid medium to form transferosomes due to inadequate proportion between medium to high concentration of EM extract and other components (phospholipid and edge activator). The reverse-phase evaporation method solved the problem by mix aqueous phase to ethanolic phase (crude extract, phospholipid and surfactant). After ethanol in mixture was completely evaporated, the transferosome vesicles containing 10% w/w of EM extract was formed. The color of formulation became darker when EM extract loading increased.

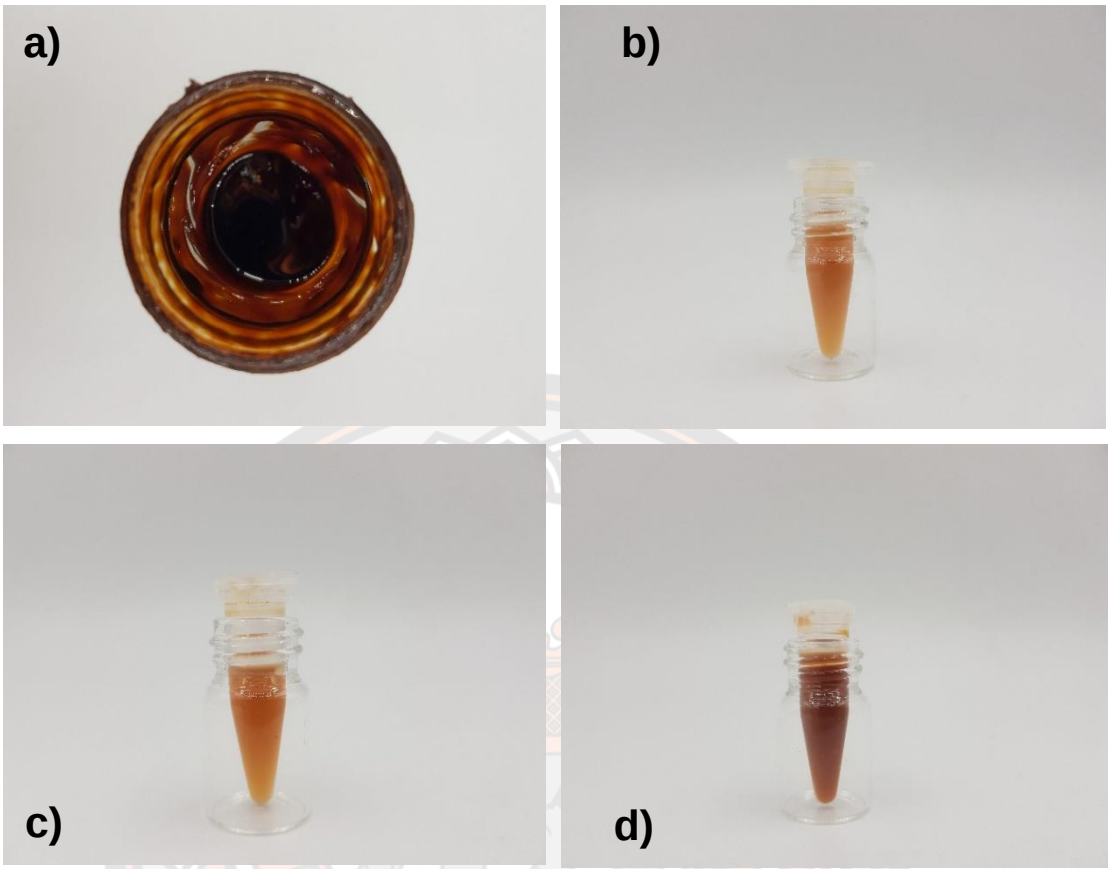


Figure 4 EM extract and transferosome formulations with different EM extract loadings: (a) EM extract (b) 2.5% w/w EM loading, (c) 5% w/w EM loading and (d) 10% w/w EM loading. SPC vesicle. EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w, Tween 80 (HLB 15.0) as edge activator and 6.8 pH, PBS as hydration medium

Physico-chemical characterization of EM extract loaded transferosomes

1. The morphology of transferosome vesicles

The EM extract was an auto fluorescence substances (Figure 5b), thereby the formation of EM extract loaded transferosomes could be determined. The large-sized transferosomes of formulation with HLB 15.0 of edge activator has been visually determined by confocal microscope (Table 4a). The most population of vesicle size was small and not clearly determined the vesicle morphology, however there were a few vesicles sized large enough to determine by confocal microscope

(Figure 5c). The morphology of vesicles with size reduction was unable to distinguish (Figure 5d).

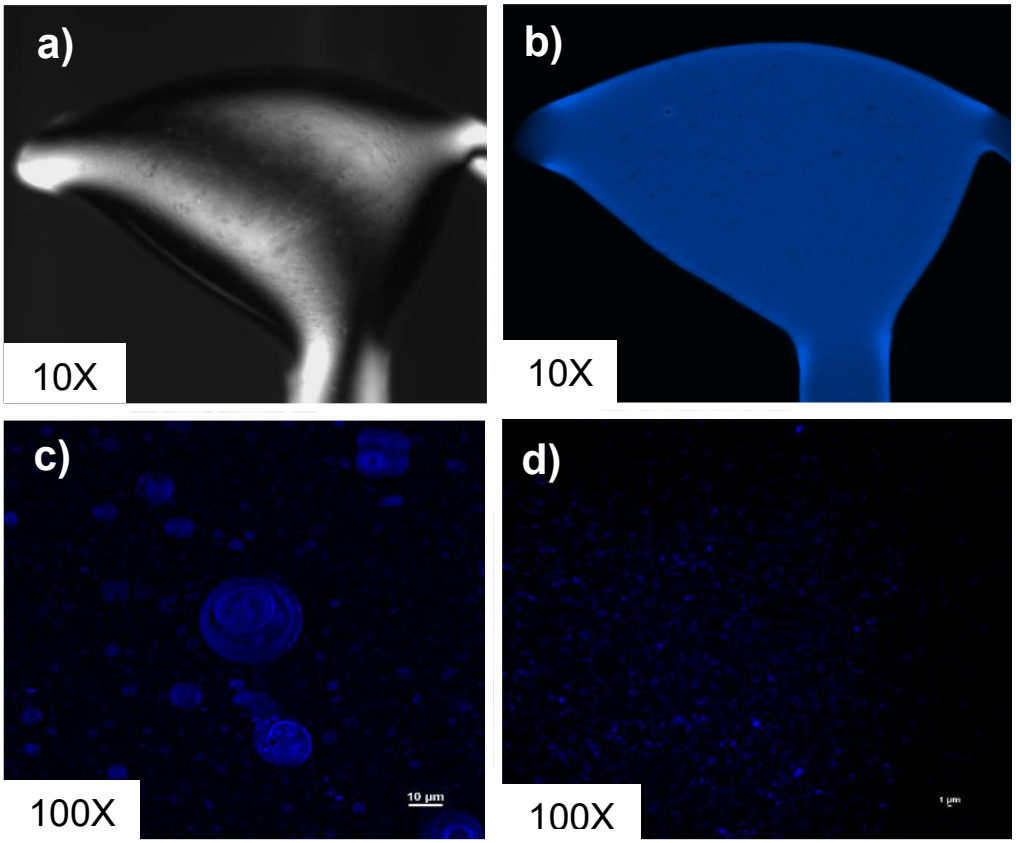


Figure 5 Morphology and characterization on confocal microscope: (a) drop of EM extract (10x), (b) The emission of EM extract (10X), Transferosomes vesicle (c) without size reduction (d) with size reduction. SPC vesicle. EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w, Tween 80 (HLB 15.0) as edge activator and 6.8 pH, PBS as hydration medium

2. The effect of phospholipid types on vesicle characterization

Based on preliminary studies, EM extract loaded transferosomes were prepared with different phospholipid types and amounts of ingredients. To obtain the formulations for in-vitro permeation studied, small vesicles and high entrapment

efficiency of transferosomes were prepared. The influencing parameters on the vesicle characteristic were determined in the follow studies.

Preparation transferosomes by the different types of phosphatidylcholines (SPC and HPC with T_m of -20°C and 50 °C, respectively). The entrapment efficiency of both phospholipids were high (~90%) and similar. The vesicle size of transferosomes increased as T_m of phospholipid increased (**Table 3**). This results were corresponded with the studies of Fumiyoshi I. and Tomoko N (42). They prepared lipid vesicle with the different type and T_m of phospholipid (acyl hydrocarbon chain C12:0, C14:0, C16:0, C18:0, C18:1 and C18:2, transition temperature 0, 23, 51, 58, -22 and -57 °C, respectively). The temperature of preparation varied from 0, 37 and 65, respectively. Their results showed the increasing vesicle size with an increase of the saturation degree of fatty acid chains. The saturated acyl hydrocarbon chains were arranged in the way that maximized the intermolecular interaction. These interactions increased T_m and decreased the fluidity of phospholipid, leading to an increase of vesicle sizes. In the opposite way, unsaturated acyl chains would probably have longer distance between the chains, resulting in fewer molecular interactions, more fluidity, and smaller vesicle sizes. This assumed the influence of T_m of phospholipid on the vesicle size. Besides the saturation degree of fatty acid chains in phospholipid, the preparation temperature also affected the vesicle size by increasing temperature with decreasing vesicle size. The preparing temperature influenced the lipid fluidity. By using higher temperature above T_m of phospholipid, the more fluidity of vesicle was obtained. These results were related to EM extract loaded transferosomes by using SPC (T_m -20 °C) and HPC (T_m 50 °C). The preparing temperatures of SPC and HPC were 25 °C and 60 °C, then the differences of working temperature and T_m were 45°C and 10°C for SPC and HPC, respectively. This indicated the fluidity of SPC on transferosome would provide smaller vesicle and less rigidity. The extrusion method (size reduction) by mini-extruder was performed. Unfortunately, the smallest size of prepared HPC vesicle was ~450 nm and still bigger than SPC vesicle due to their high rigidity. In addition, the skin permeability of SPC and HPC transferosomes were performed. However, the polydispersity index of transferosomes of HPC was greater than SPC. This meant higher size distribution for HPC vesicle and this implied to physical stability of formulation due to the aggregation. From the result of

phospholipid types, the SPC was more suitable to prepared as topical formulation due to its low rigidity providing smaller vesicle size and lower tendency of vesicle aggregation (66, 67).

Table 3 The effect of phospholipid type to vesicle size, zeta potential and entrapment efficiency (Mean±SD, n=3)

Results	Phospholipid types	
	SPC T_m -20 °C	**HPC T_m 50 °C**
Vesicle size (nm)	346.40±19.40	1897.50±88.38
Polydispersity index	0.34±0.02	0.59±0.08
Zeta potential (-mV)	4.13±0.68	6.24±0.40
Entrapment efficiency(%)	91.53±1.52	91.1±1.56

SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w. Span 80 (HLB 4.3) as s edge activator and 6.8 pH, PBS as hydration medium. No size reduction.

3. The effect of EM extract to phospholipid ratios on vesicle characterization

The influences of proportions of EM extract to SPC in formulations were investigated. the entrapment efficiency and vesicle size were increased with increasing phospholipid part (1:2 to 1:4), due to more layers of vesicle and more entrapment of EM extract (**Table 4**). The ratio of 1:2 was chosen to prepared as topical formulation instead of 1:4 concerning about higher EM loading. The formulation with 1:1 of EM extract to SPC was not further studied because this formulation was heterogeneous appearance. This could be attributed to incomplete dispersion of EM extract in hydration medium and resulted in the segregation of components due to inadequate amount of phospholipid. Phospholipid was the important ingredient used to increase the solubility and dispersed the EM extract in medium. These results showed the influences of EM extract and SPC phospholipid on the formulation, which might probably affect to physical appearance, stability and efficacy of the formulation.

Table 4 The effect of EM extract and phospholipid ratio to vesicle size, zeta potential and entrapment efficiency (Mean ± SD, n=3)

Results	EM extract: SPC ratio (w/w)		
	1:1	**1:2**	**1:4**
Vesicle size (nm)	N/A	346.40±19.40	434.10±22.63
Polydispersity index	N/A	0.34±0.02	0.34±0.01
Zeta potential (-mV)	N/A	4.13±0.68	2.98±0.29
Entrapment efficiency(%)	N/A	91.53±1.52	97.94±2.21

SPC vesicle with EM extract loading 2.5% w/w, edge activator: SPC 15:85% w/w. Span 80(HLB 4.3) as surfactant and 6.8 pH PBS as hydration medium. No size reduction.

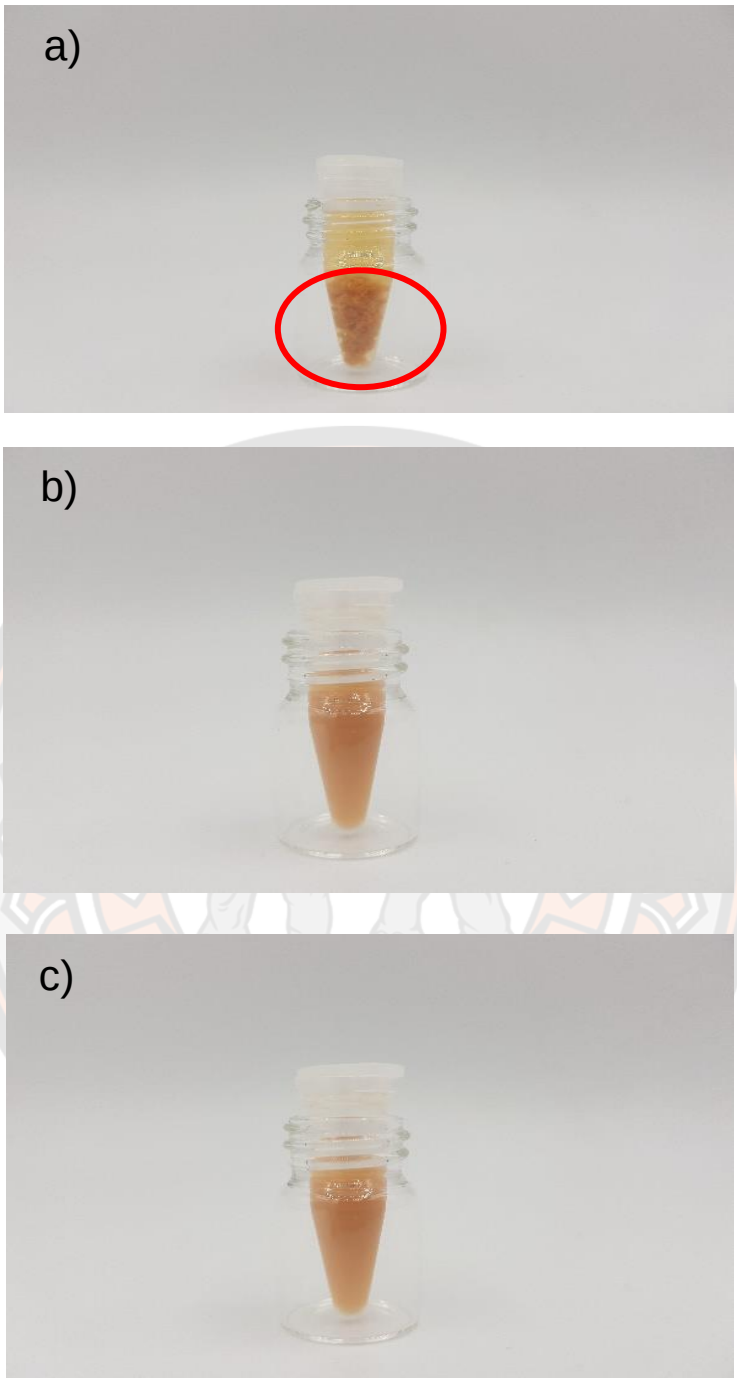


Figure 6 The physical appearance of transferosomes with different ratios of EM extract and SPC(w/w): (a) ratio 1:1, (b) ratio 1:2 and (c) ratio 1:4. The red circle on a) was the segregate components

4. The effect of HLB of edge activator on vesicle characterization

Edge activator (surfactant) played the important roles in transferosomes properties with improving the flexibility, drug entrapment efficiency and other vesicle characteristics (54). The HLB of edge activator also affected to vesicle characteristic. The determine the effects HLB of edge activator on transferosome properties the HLBs were varied from 4.3 to 15.0. The entrapment efficiencies of formulations were similar (~90%). However, the vesicle size increased from ~340 to ~990 nm with increasing HLB (**Table 5**). This could probably be explained by an increase of HLB leading to an also improvement of solubility/dispersibility of phospholipid and EM extract to hydration medium (aqueous). The phospholipid possibly formed the vesicle that entrapped more aqueous part, so the vesicle size was enlarged. To investigated this hypothesis, the blank vesicles (without EM extract) with the same method were prepared by the varied HLBs from 4.3 to 15.0 and measured the osmolarity of dispersion medium of blank transferosomes. The size of blank vesicle increased with increasing of HLB and osmolarity of dispersion medium, which increased from 245 to 410 mOsm. Therefore, the increase of surfactant HLB could improve the solubility/dispersibility of water components. This supposed the vesicles were capable to entrap more aqueous part and enlarge the vesicle size. The vesicle size was increased as HLB of edge activator increased. However, the formulation with polydispersity index over 0.3 indicated wide range of size distribution and this was not suitable to incorporate in the formulation. Wide size distribution probably effected to the stability of formulation and inconsistency of applied dose. A method for size and polydispersibility index reduction was required.

Table 5 The effect of HLB of edge activator to vesicle size, zeta potential and entrapment efficiency (Mean±SD, n=3)

Results	HLB value				
	4.3	**6.98**	**9.65**	**12.33**	**15.0**
Vesicle size (nm)	346.40±19.40	505.00±38.35	622.96±14.18	736.40±33.4	882.70±56.08
Polydispersity index	0.34±0.02	0.35±0.01	0.36±0.02	0.41±0.02	0.35±0.02
Zeta potential (- mV)	4.13±0.68	4.64±1.19	4.63±1.93	4.29±0.80	5.74±2.94
Entrapment efficiency (%)	91.53±1.52	91.37±1.19	91.05±0.93	90.33±0.61	91.12±0.38

SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w and 6.8 pH, PBS as hydration medium and no size reduction.

The vesicle size and polydispersity index of transferosomes were reduced by extrusion method (0.4 and 0.1 µm pore size). After extrusion, the vesicle size of each formulation were in the same range, even though the HLB of edge activator increased. The polydispersity indexs were less than 0.3indicating monodisperse vesicles (**Table 6**). These results of HLB surfactants were in agreement with the studies of Jain et al. (55) and Al Shuwailli et al. (56)

In this studied, transferosomes were reduced the vesicle size and polydispersibility index by mini-extruder with 0.4 µm pore size prior the deformability determination of vesicle. Deformability determinations of the formulation with HLB of edge activators (4.3, 9.65 and 15.0) compared with initial vesicle size ~200 nm were performed using mini-extruder with 0.1 µm membrane pore size. All vesicle sizes of formulations were ~100 nm. The deformability of all formulations with different HLB were similar (~50%). Then, these HLB formulations

with similar vesicle characteristics (e.g. size, % EE and deformability) were further determined and compared the skin permeability.

Table 6 The effect of size reduction by mini-extruder (extrusion method) to vesicle size, zeta potential and entrapment efficiency (Mean ± SD, n=3)

HLB	Membrane pore size					
	0.4 µm			0.1 µm		
	Vesicle size (PDI)	Zeta potential (-mV)	Entrapment efficiency (%)	Vesicle size (PDI)	Zeta potential (-mV)	Entrapment efficiency (%)
4.3	209.00±17.34 (0.24±0.01)	3.96±0.52	91.65±1.40	101.70±12.50 (0.08±0.01)	4.20±2.36	91.00±1.07
9.65	192.70±15.39 (0.13±0.06)	4.75±1.21	90.74±1.42	98.30±6.25 (0.13±0.01)	4.52±0.89	91.80±0.65
15.0	204.90±5.34 (0.12±0.01)	3.84±2.24	92.2±0.49	97.70±10.82 (0.08±0.06)	4.90±0.54	92.10±0.49

SPC vesicle with EM extract loading 2.5% w/w, EM extract:SPC 1:2 w/w, edge activator:SPC 15:85% w/w and 6.8 pH, PBS as hydration medium.

5. The effect of edge activator concentration on vesicle characterization

Besides the HLB of edge activator, the concentration of surfactant probably also affected to vesicle characters. Gupta et al. (40) studied the vesicle characteristics of transferosomes with sertraline, by increasing edge activator concentration from 5%-25% w/w to phospholipid. The vesicle size and entrapment efficiency of transferosomes increased with increasing edge activator in formulation up to ~20% edge activator. This could be attributed to an improvement of the sertraline solubility in lipid layer of vesicle, however the vesicle size and entrapment efficiency started drop down with the formulation containing 25% edge activator. Increasing amount of edge activator might lead to the formation of micelles instead of transferosomes. Micelles were small vesicles having lower entrapment and flexibility compared to transferosomes. Their studies related to the results of Jain et al (55), the

reduction of vesicle size with increasing the concentration of edge activator above 15% w/w was shown. The formation of micelles was their explanation in this study. EM extract loaded transferosomes with increasing concentrations of edge activator from 5%-20% w/w to phospholipid were prepared. No effect of concentration of edge activator on the entrapment efficiencies of all formulations was presented (**Table 7**). This could probably be explained that the amount of Compound 1 in EM extract was pretty low and most of them were completely entrapped by lipid bilayer of transferosomes. The vesicle size remained the same while increasing the concentration of surfactant. Then, the concentration of edge activator from 5%-20% w/w had no effect on vesicle characteristic in this study. It seemed that the proportion of edge activator to phospholipid played a crucial role on vesicle systems (transferosomes vs micelles), deformability of vesicle skin permeability of drug. Diclofenac loaded transferosomes which varied the types and concentration of edge activator were prepared and studied El Zaafarany G.M et al. (68). The edge activator: phosphatidylcholine (15:85 % w/w) possessed the optimum deformability and drug release. Their results suggested the concentration of edge activator either low or high would affect the vesicle characteristic and skin permeability. Low concentration of edge activator induced low flexibility and low skin permeability. High concentration of edge activator (> 20-25%) could induce the forming of micelles in bilayer, resulting in a decrease in entrapment efficiency. The effect of micelles formation on size and rigidity of vesicle would resulted in low skin permeability (68). From the previous studies of Jain et al. (55), Zaafarany G.M et al. (68) and Gupta et al. (40), the concentration of edge activator effected to vesicle characteristic such as size, deformability and skin permeability. Therefore, the preparation of EM extract loaded transferosomes with 15:85 % w/w of edge activator:phosphatidylcholine was reasonable to prepared the formulation.

Table 7 The effect of edge activator concentration to vesicle size, zeta potential and entrapment efficiency (Mean ± SD, n=3)

Results	SPC : Edge activator (HLB 15.0, % w/w)			
	95:5	**90:10**	**85:15**	**80:20**
Vesicle size (nm)	779.00 ± 15.60	816.00 ± 22.60	882.73±56.08	957.00 ± 45.30
Polydispersity index	0.34 ± 0.02	0.32 ± 0.01	0.35±0.02	0.34 ± 0.02
Zeta potential (-mV)	5.02 ± 0.52	5.29 ± 1.33	5.74±2.94	5.24 ± 0.83
Entrapment efficiency(%)	92.3 ± 0.45	92.5 ± 1.13	91.12±0.38	92.04 ± 0.87

SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, 6.8 pH PBS as hydration medium and no size reduction.

6. The effect of hydration mediums on vesicle characterization

To improve the convenience of using the topical formulation, the addition of thickening agent into the formulation to provide the adhesion skin for better drug absorption was done. Carbomer (Carbopol®) was used as a viscosity inducing agent. Unfortunately, this gelling agent had low resistance to ion, so deionized (DI) water was used as hydration medium instead of 0.05 M phosphate buffer solution (PBS). The prepared transferosomes with DI water showed similar vesicle size and entrapment efficiency as PBS. The similarity of transferosomes hydrated with both mediums were shown in **Table 8**.

Table 8 The effect of hydration medium to vesicle size, zeta potential and entrapment efficiency (Mean ± SD, n=3)

Results	Hydration medium	
	pH 6.8 phosphate buffer	Deionized water
Vesicle size (nm)	204.90±5.34	211.20±9.1
Polydispersity index	0.12±0.01	0.23±0.03
Zeta potential (mV)	-3.84±2.24	-5.79±1.28
Entrapment efficiency (%)	92.2±0.49	92.43±0.84

SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w, Tween 80 (HLB 15.0) as edge activator. Size reduction method by mini-extruder with 0.4 µm pore size membrane.

In vitro permeation study

The in vitro permeation studies of all transferosomes were performed using the artificial skin (Strat M®). Strat M® was the synthetic membrane composed of polymeric layers and embed lipid component, which mimic to human skin. All formulations were successfully delivered EM extract across the skin (Strat M®). The correlation (R^2) of Strat M® and human skin was more than 0.9 (43). This demonstrated the permration similarity of Strat M® and human skin. The Strat M® membrane has provided consistent results, reproducibility, availability and minimum variability with human skin (43, 69). It was reasonable to used Strat M® instead of human skin in this experiment. For this study, the influencing parameters on skin permeability of transferosome vesicles (phospholipid types, HLB of surfactant and vesicle size) were determined.

1. The effect of phospholipid types on skin permeability

The previous result (Table 3) showed the different effects between phospholipid types on the rigidity of transferosomes. The larger vesicle size and more rigid of transferosomes from HPC compared to SPC were presented. This result

would probably relate to the skin permeability. The relations between flexibility and skin permeability of transferosomes were investigated. HPC transferosomes has been prepared and reduced the vesicle size by extrusion method (cut of size was 0.4 μm membrane pore size), but unfortunately the smallest vesicle size was only ~450 nm due to the viscous formulation obtained. The skin permeability of HPC tranferosomes were compared to SPC transferosomes with the vesicle size ~900, ~350 nm (without size reduction) and ~200 nm (with size reduction). The results on skin permeability were shown in Figure 7, HPC transferosomes had the lowest skin permeability compared to solution and SPC transferosomes, even though SPC tranferosomes had larger vesicle size. This could been probably contributed to the higher rigidity vesicle and difficulty of HPC transferosomes to permeate the skin. Moreover, the temperature during skin permeability test (~32 $^{\circ}$C) was above T_m of SPC. Therefore, the lipids were in liquid crystalline state having fluidity and flexibility to pass through the membrane. While HPC had a high T_m (50 $^{\circ}$C) above the experiment temperature, HPC transferosomes were in the gel state and rigid vesicles. This would reduce the skin permeability of transferosomes. Then, SPC transferosomes vesicles provided better skin permeability compared to HPC transferosomes due to their fluidity and flexibility. The EM extract loaded transferosomes for topical formulation by using SPC as carriers was reasonable (67).

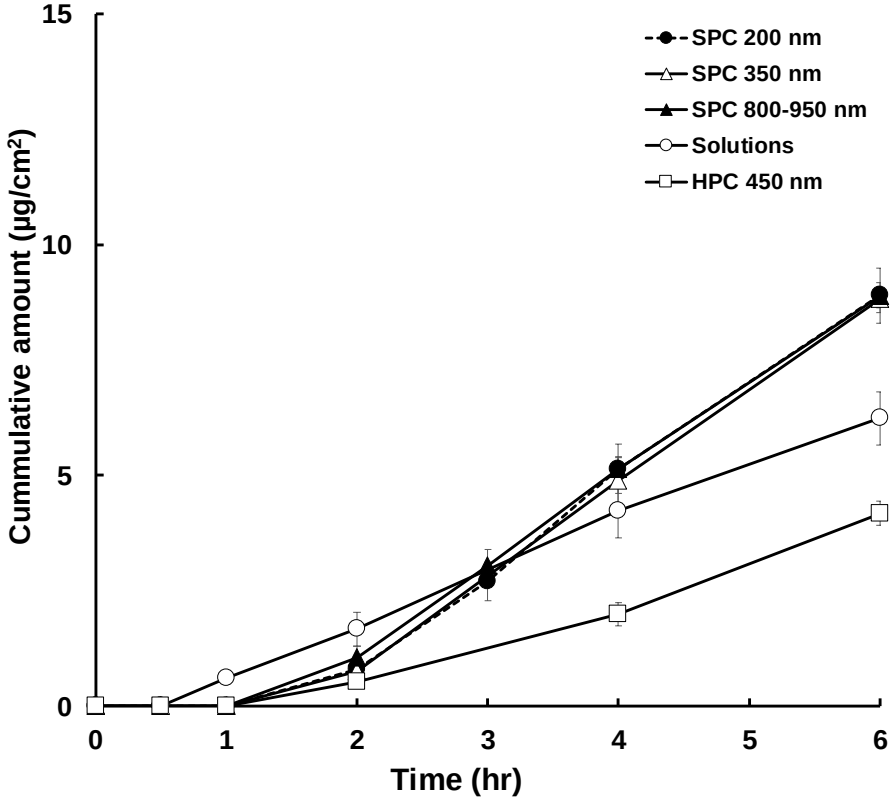


Figure 7 The skin permeability comparison of SPC and HPC transferosomes vesicle. EM extract loading 2.5% w/w, EM extract:SPC 1:2 w/w, edge activator:SPC 15:85% w/w. Span80 (HLB 4.3) as edge activator of SPC 200, 350 nm and HPC. For Tween 80 (HLB 15.0) as edge activator of SPC 800-950 nm ,6.8 pH PBS as hydration medium (Mean±SD, n=3)

2. The effect of HLB of edge activators to skin permeability

The edge activator had an important role on the flexibility and skin permeability of transferosomes. From the study of El Zaafarany G.M et al. (68), the types and ratios of edge activator were varied. Their vesicle composed of phosphatidylcholine and Tween 80 as edge activator; concentration of edge activator: phospholipid as 15:85% w/w provided highest flexibility and drug release. Their results probably confirmed the drug release relating to vesicle flexibility (68). However, in this skin permeability studies of EM extract loaded transferosomes which

varies the HLB edge activators of 4.3, 9.65 and 15.0. The skin permeabilities of these formulations were comparable. This might be due to the similarity of vesicle size (~100 nm) and deformability of each formulation (~50%) (**Table 6**). This result indicated rising of HLB (Figure 8) had no effect on skin permeability.

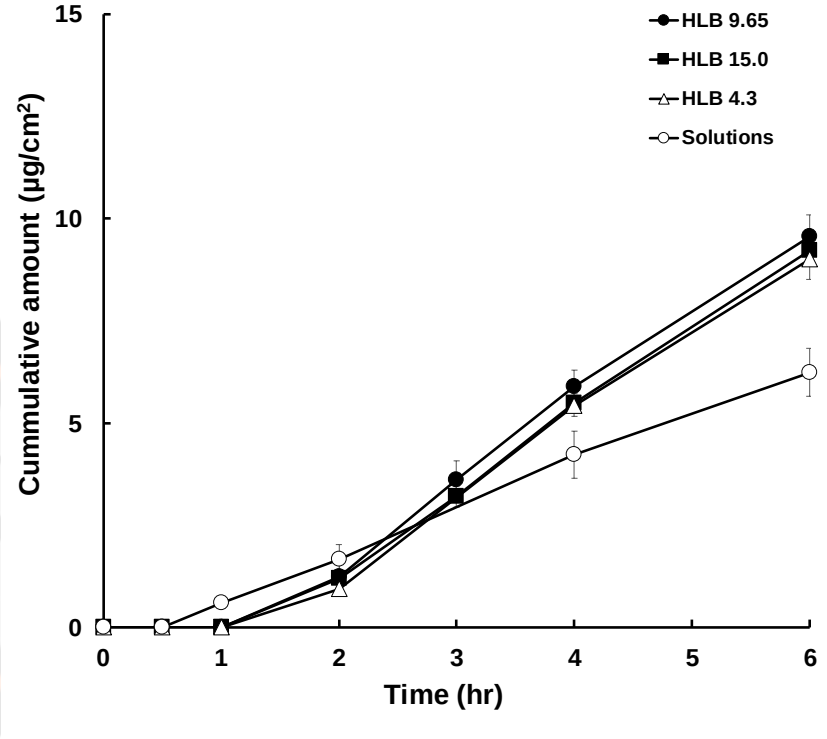


Figure 8 Effect of HLB to skin permeability. SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w. The vesicle size and entrapment efficiency of all formulation ~100 nm and ~90%, respectively. (Mean±SD, n=3)

3. The effect of transferosomes vesicle size to skin permeability

The explanatory mechanism which elucidated the relationship between the skin permeability of transferosomes and fluidity of phospholipid was described. The components of vesicle would disturb the structure of skin by adsorption /fusion on surface of membrane leading to an alteration of lipid properties in membrane. This probably improved the fluidity of lipid and components in membrane. Then vesicle could permeate easily into the deeper layers of the skin by intercellular and

intracellular pathway. The temperature during skin permeability study (~32 ˚C) was higher than T_m of SPC (-20 ˚C), this would improve fluidity and flexibility of vesicles [67]. Since the flexibility and deformability of transferosomes have been shown, vesicles were able to deform themselves and pass through narrow construction by 5-10 times narrower than their own diameter (57). Therefore, skin permeability of formulations still similar even increased the vesicle size to ~900 nm. This study indicated that transferosomes with size ranged from ~100 to ~900 nm did not affect skin permeability (Figure 9). Anyway, the formulations with vesicle size ~350 nm (HLB 4.3) and ~900 nm (HLB 15.0) were not further developed due to wide vesicle size distribution (PDI > 3.0). This probably affected the stability and uniformity of application dose

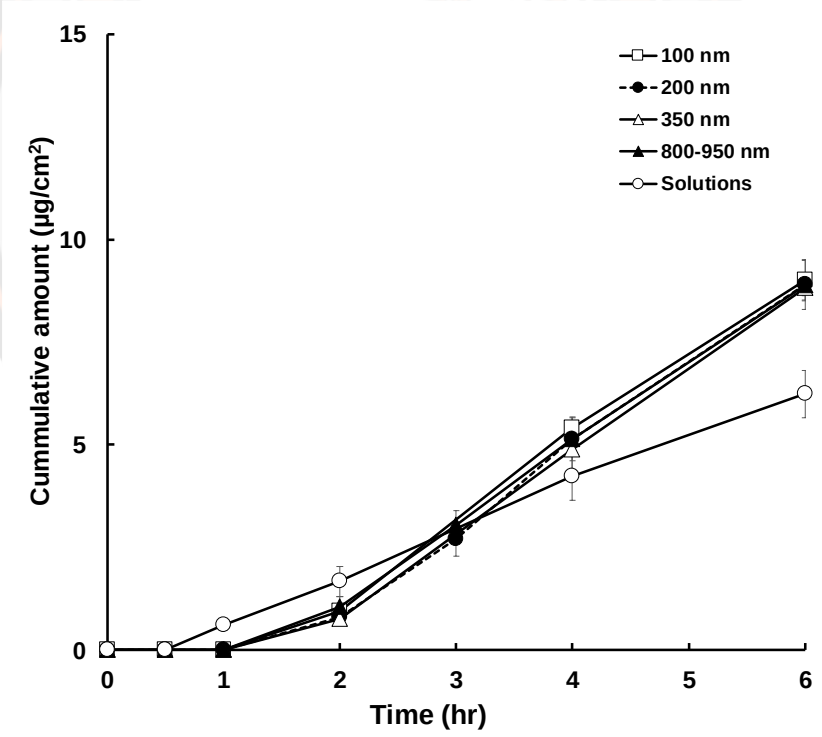
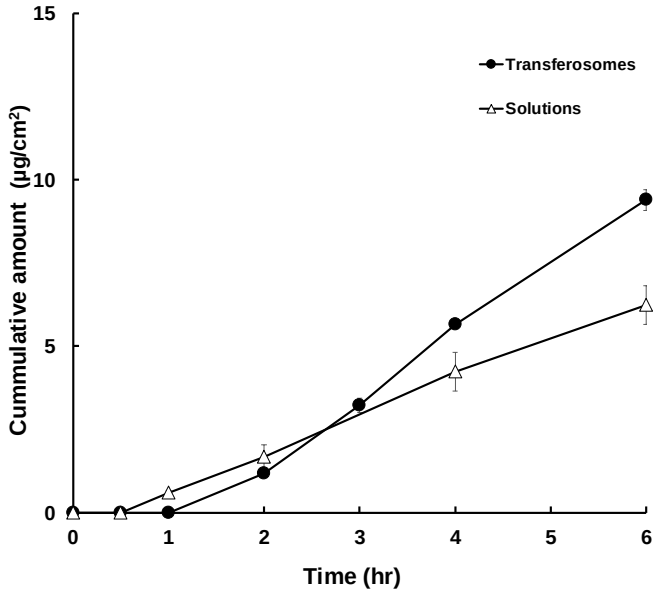


Figure 9 Effect of vesicle size to skin permeability. SPC vesicle with EM extract loading 2.5% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w. The entrapment efficiency of all formulation ~90%. Edge activator HLB 4.3 for vesicle size 100-350 nm and HLB 15.0 for 800-950 nm. (Mean±SD, n=3)

The previous results from surfactant HLBs and vesicle sizes showed the skin permeability of transferosomes was better than solutions (Figure 10a). To clarify this result, the skin penetration mechanism would be described. The mechanism could be explained by a disruption of skin structure by the properties of formulation. This led to an increase in skin permeability. The mechanisms of EM extract-transferosome delivery across the skin would be intercellular and intracellular pathway due to the components and flexibility of vesicle. Compare to transferosomes, solutions mainly delivered EM extract by intracellular pathway. Lipophilic Compound 1 in the solutions diffused mainly into the lipid of membrane (Strat-M®). This finding was related to the results in Figure 10b Higher amount of Compound 1 in EM extract was left on donor and entrapped in the membrane compared to transferosomes formulations. By the way transferosomes had a bit longer lag time (~1.0 hr) than solutions (~ 0.5 hr) to permeate through the skin. Slower skin permeation of transferosomes might be from the lower disruption to skin barrier. The components of transferosomes would contributed effect on the penetration mechanism. The skin enhancers (phospholipid and edge activators) had less disruption to the skin compared to solutions (hydro-alcoholic solvent as skin enhancer). In this study, transferosomes with high entrapment efficiency of EM extract (~90%) were prepared, which meant less unentrapped extract was about ~90%. This probably took time to un-stabilized and deliver EM extract loaded transferosomes across membrane. Increasing unentrapped Compound 1 by increasing EM extract loading and skin enhancer would be further determined. This might possibly shorten the lag time of skin permeability.

a)



b)

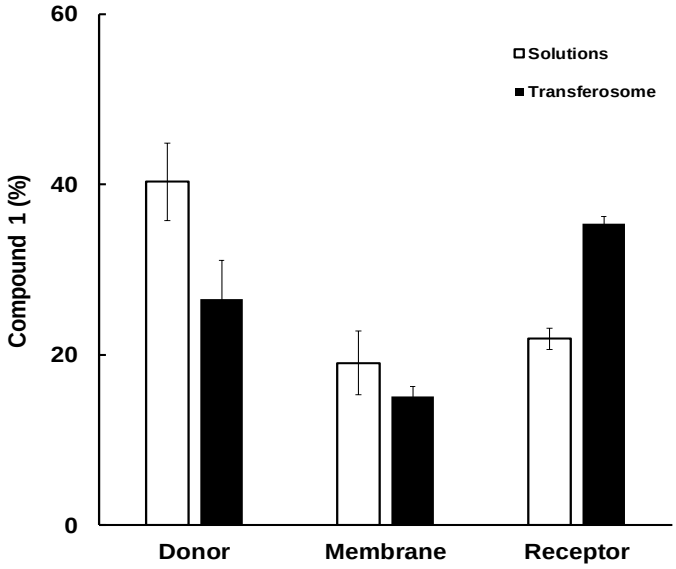


Figure 10 The comparison of skin permeability and amount of Compound 1 residue in each compartment from Franz's diffusion cell of transferosomes and solution; a) Cumulative amount of Compound 1 in transferosomes and solutions b) residue of Compound 1 at 6 hours in solutions and transferosomes (Mean±SD, n=3)

4. The effect of EM extract loading in transferosomes to skin permeability

To overcome lag time problem of transferosomes, an enlarged amount of free Compound 1 (un-entrapped) by increasing plant extract loading was performed. Hypothesis of the experiment, the lag time of permeation would be reduced by increasing the EM extract loading, then more amount of un-entrapped Compound 1 would be presented. From the result of the experiment, the AUC peak of 2.5, 5.0 and 10% w/w EM extract loading started to reveal at 2 hrs, 1 hr and 0.5 hr, respectively. This implied shorten lag time with increasing EM extract loading (Figure 11). The other explanation of this phenomenon was an increase of phospholipid and edge activator concentration which could promote the skin permeability. According to the ratio of ingredients of each formulation with same total amount of formulation (10 g). Each formulation preparing with same ratio of EM extract: SPC (1:2 w/w) and edge activator: SPC (15:85% w/w). The concentration of SPC and edge activator increased as concentration EM extract increased from 2.5%-10% w/w. SPC and edge activator increased from 5-20% w/w and 0.88-3.53 % w/w, respectively. Phosphatidylcholine and surfactant were able to disturbed the structure of membrane and improve the skin permeability of EM extract. Increasing the concentration of SPC and edge activator could possibly increase the skin permeability of EM extract. This phenomenon was also found by Kim C et al. (70), caffeine was entrapped in transferosomes with the concentration of phosphatidylcholine from 1-4% w/w. The permeation of caffeine was increased as concentration of phosphatidylcholine increased (70). Valjakka-Koskela R. et al. (71) studied the skin penetration of naproxen by varies the concentration of phospholipid (0.4-4% w/w) and others skin enhancers. The skin penetration of naproxen was increased as concentration of phospholipid increased (71). From the result, an increase of EM extract loading and concentration of skin enhancer (SPC and edge activators) could probably reduce the lag time of skin permeability.

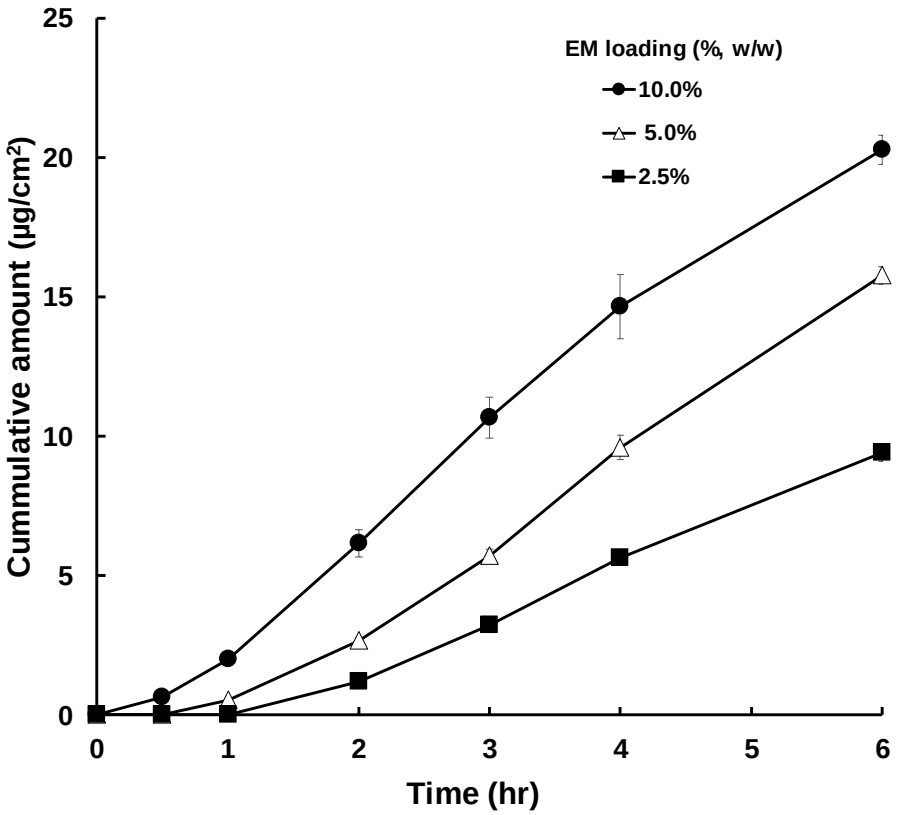


Figure 11 Effect of crude extract loading to skin permeability. SPC vesicle with EM extract loading 2.5, 5.0 and 10.0% w/w, EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w. The entrapment efficiency of all formulation ~90%. Vesicle size of 2.5-5.0 and 10.0 % w/w EM extract loading were ~200 and ~350, respectively. (Mean±SD, n=3)

5. The skin permeability of transferosomes incorporated in suspensions and gels

To improve the efficacy of formulations by increasing absorb time on the skin, the transferosomes were incorporated in a formulation having good adhesive property on the skin. Carbomer (Carbopol® Ultrez 21) was used to the transferosome gels due to its properties concerning about viscosity, adhesion. and ion tolereance, Deionized water was used as hydration medium instead of phosphate buffer, owning to ion tolerance of carbopol. The skin permeability of transferosomes dispersed in PBS, DI water and gel base were compared (Figure 12). Solution of EM extract showed faster (shorter lag time) skin permeability but further permeability became

lower than transferosomes. No difference in skin permeability of transferosome between suspensions and gels was shown. Carbopol® as gel base could improve skin adhesion but it did not retard skin permeability.

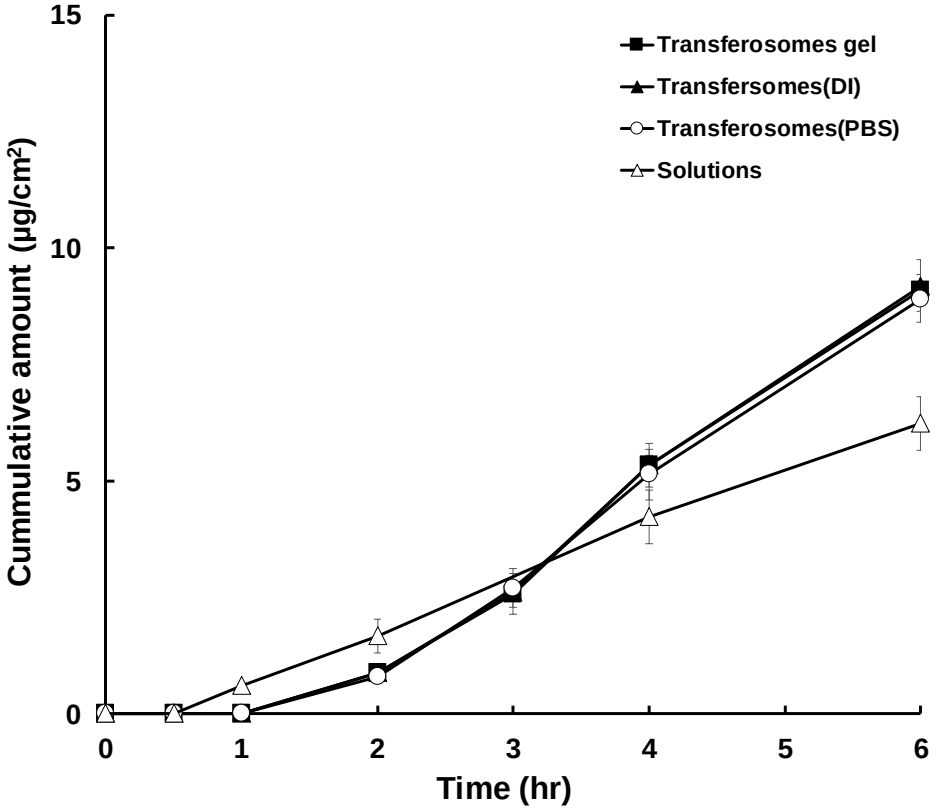


Figure 12 Comparing the skin permeability of transferosomes gels (0.5% w/w, Carbopol®) and transferosomes with the different dispersion medium of DI water and pH 6.8 PBS, respectively. EM extract loading 2.5% w/w for all formulation. Transferosomes composition and ratio; EM extract: SPC 1:2 w/w, edge activator: SPC 15:85% w/w. Tween 80(HLB 15.0) as edge activator (Mean±SD, n=3)

Stability test

1. Vesicle characterization and formulation appearance

Physical stability

The vesicle characterizations of both storage conditions for 12 weeks were investigated. The small aggregation of transferosomes was presented. The aggregation of transferosomes could be explained by low repulsion force due to low zeta potential representing the charge on vesicle surface (74). However, the formulations were fully re-dispersed after shaking. The vesicle characteristic has been determined, vesicle size, zeta potential and size distribution were comparable during 12 weeks storage (Table 9). This probably polar head of vesicle interacted with water and resulting a shell of water molecule that prevent aggregation of vesicle (72, 73). The repulsive force was not strong enough to prevent the vesicle aggregation. The formulation might fully aggregate and probably not re-dispersed for storage in longer duration.

Table 9 The particle size(nm) and zeta potential(mV) of formulation for 12 weeks in different conditions (Mean±SD, n=3)

	Storage temperature 40°C							
	Week							
Transferosomes	**0**	**1**	**2**	**4**	**6**	**8**	**10**	**12**
Vesicle size(nm)	213.00±6.08	213.00±6.24	209.67±6.11	210.87±6.02	217.47±3.37	207.67±3.79	205.37±5.05	211.27±6.63
Zeta potential (mV)	-4.21±1.20	-3.30±1.81	-3.05±2.12	-4.32±1.08	-3.41±1.65	-4.98±2.51	-3.20±1.83	-3.81±2.31
PDI	0.13±0.01	0.17±0.03	0.17±0.05	0.16±0.02	0.18±0.01	0.20±0.04	0.29±0.02	0.29±0.02

Storage temperature 4°C								
	Week							
Transferosomes	**0**	**1**	**2**	**4**	**6**	**8**	**10**	**12**
Vesicle size(nm)	213.00± 6.08	216.67± 10.97	204.60± 2.17	206.93± 5.29	207.83± 2.74	209.30± 10.54	205.93± 6.13	206.80± 3.68
Zeta potential (mV)	-4.21±1.20	-3.21±1.52	-4.20±1.89	-4.32±1.41	-3.25±1.89	-3.51±2.13	-2.87±1.02	-3.32±1.75
PDI	0.13±0.01	0.11±0.01	0.19±0.02	0.15±0.01	0.16±0.02	0.16±0.01	0.17±0.03	0.16±0.03

Chemical stability

The quantitative determination of Compound 1 entrapped in transferosomes showed good stability after 12 weeks storage in refrigerator (4°C) and oven (40°C). No change of Compound 1 amount after storage in both conditions compared with fresh preparation (Figure 13**)**. That could imply that Compound 1 was stable at both conditions in this study.

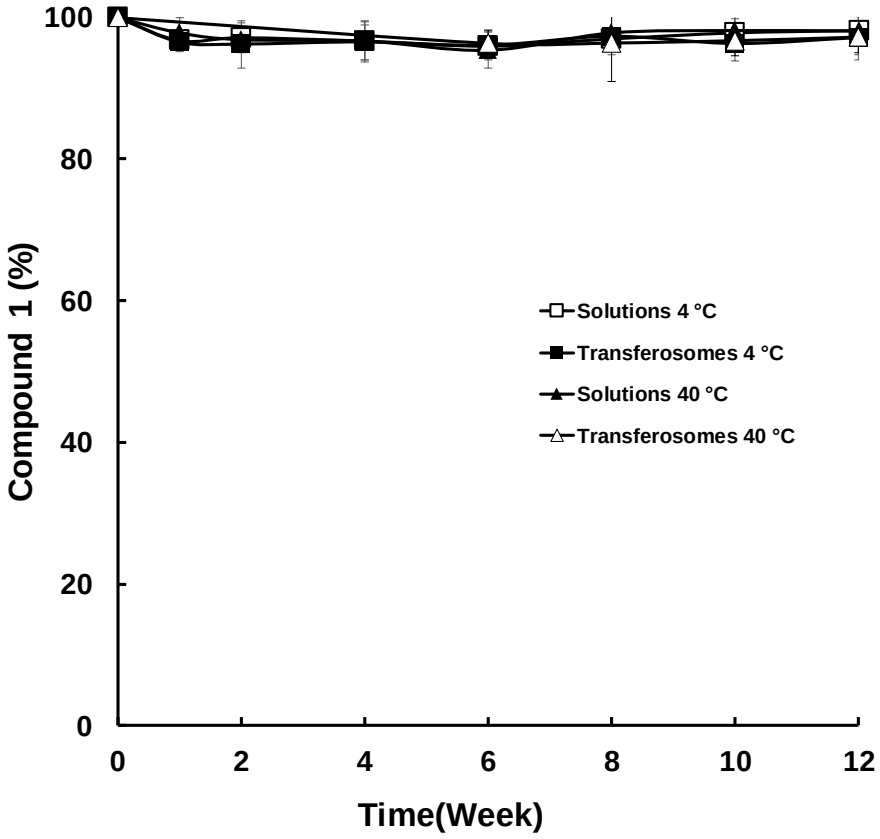


Figure 13 Stability of Compound 1 that contained in transferosomes with the different storage conditions of 4˚C and 40˚C (mean±SD, n=3)

2. Skin permeability

For skin permeability test of formulation during storage of both condition. The comparison result of freshly prepared and week 10 were similar (Figure 14), F2 of results were over 80% and F1 less than 10% for both storage condition (4˚C and 40˚C) (Table 10). According to the result, both storage conditions for 10 weeks had no effect on skin permeability of transferosomes. This because the vesicle characteristic remained the same

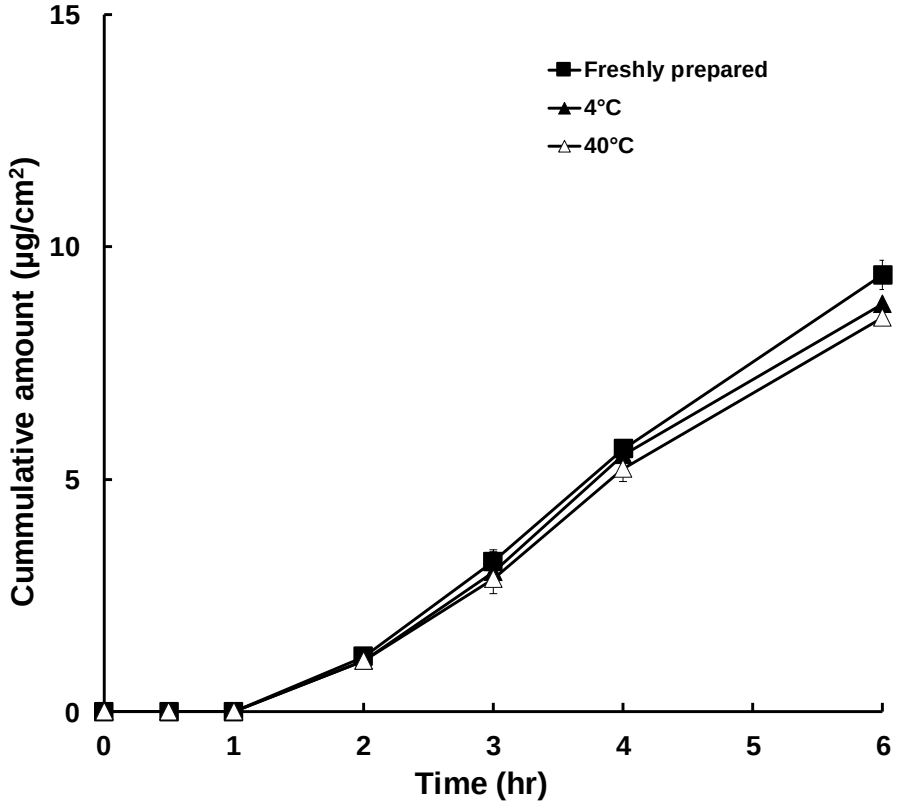


Figure 14 Skin permeability of freshly prepared transferosomes and 10 weeks later after storage with the different condition of temperature: 4 °C and 40°C (Mean±SD, n=3)

Table 10 The similarity of drug release by transferosomes at week 0 and week 10 in the different storage conditions

Transferosomes	F1	F2
Storage temperature 4°C	5.4	99.2
Storage temperature 40°C	9.2	98.1

CHAPTER V

CONCLUSION

In conclusion, the transferosomes with small vesicle size, high entrapment efficiency and good deformability were successfully developed. SPC with low T_m gave small vesicle size due to low rigidity of phospholipid. The suitable ratio of EM extract to SPC was 1:2 w/w. Vesicle size was increased as concentration of edge activator increased. Different hydration mediums had no effect on zeta potential and vesicle size. Increasing HLB of edge activators affected vesicle size. The transferosomes after extrusion showed ~50% deformability. The transferosomes with different HLBs of edge activator, similar vesicle size and deformability would provide similar skin permeability. To increase efficacy of EM extract by extended absorption, Carbopol® gel base was added into the formulations for skin adhesion. Transferosomes could improve skin permeability and provide better drug release than solutions. All SPC transferosomes formulation (HLB of edge activator 4.3,9.65 and 15.0) with 2.5% EM extract loading had similar skin permeability with lag time ~1.0 hour. The lag time was shorten by increasing drug loading due to enlarged amount of unentrapped Compound 1 and skin enhancer (phosphatidylcholine and edge activator).

The transferosomes and solutions were physically and chemically stable in both storage conditions of 4 and 40 °C for 12 weeks. For the physical stability of transferosomes, small aggregate appeared but they could be fully re-dispersed after shaking. No changes in vesicle properties and skin permeability after storage were presented.

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