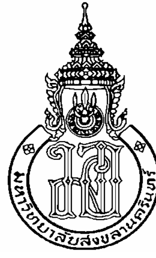

Development of Rice Bran Oil Nanosome for Using in Cosmetic

Pariporn Maharachatahtakul

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Cosmetic Sciences (International Program)

Prince of Songkla University

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Thesis Title	Development of Rice Bran Oil Nanosome for Using in Cosmetic
Author	Miss Pariporn Maharachatahtakul
Major Program	Cosmetic Sciences (International Program)

Major Advisor	**Examining Committee :**
....	....Chairperson
(Dr. Thanaporn Amnuaikit)	(Asst. Prof. Dr. Prapaporn Boonme)
	....Committee
Co-advisor	(Dr. Thanaporn Amnuaikit)
....	....Committee
(Asst. Prof. Dr. Sirirat Pinsuwan)	(Asst. Prof. Dr. Sirirat Pinsuwan)
	....Committee
	(Asst. Prof. Dr. Kulthida Vongbupnimit)
	....Committee
	(Dr. Sarunyoo Songkro)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Cosmetic Sciences (International Program)

…..

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์	การพัฒนาผลิตภัณฑ์นาโนโซมของน้ำมันรำข้าวเพื่อใช้ในทาง เครื่องสำอาง
ผู้เขียน	นางสาวภาริภร มหารชตะธกุล
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บทคัดย่อ

รำข้าวเป็นองค์ประกอบของข้าวที่มาจากกระบวนการผลิตข้าว เมื่อนำรำข้าวมาแปรรูปจนได้น้ำมันรำข้าว ซึ่งในน้ำมันรำข้าวนี้พบว่ามีสารต้านอนุมูลอิสระตามธรรมชาติหลายชนิด อย่างเช่น gamma-oryzanol, tocopherol และ tocotrienols ดังนั้นจึงได้นำนาโนเทคโนโลยีเข้ามาเพื่อเตรียมน้ำมันรำข้าวให้อยู่ในรูปนาโนโซมเพื่อพัฒนาความสามารถในการซึมผ่านผิวหนัง ในการวิจัยนี้เป็นการศึกษาการพัฒนาผลิตภัณฑ์นาโนโซมของน้ำมันรำข้าวเพื่อใช้ในทางเครื่องสำอาง โดยเริ่มจากการนำน้ำมันรำข้าวไปทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH radical scavenging assay พบว่าน้ำมันรำข้าวมีค่าความเข้มข้นที่สามารถกำจัดอนุมูลอิสระได้ 50% (EC_{50}) เท่ากับ 4.396 ± 0.282 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้ได้พัฒนาความสามารถในการซึมผ่านผิวหนังของน้ำมันรำข้าวโดยเตรียมให้อยู่ในรูปแบบนาโนโซมของน้ำมันรำข้าวด้วยวิธี reverse phase evaporation ซึ่งใช้สารไขมัน คือ soybean phosphatidylcholine (SPC) และสารตัวเติม 3 ชนิด คือ Tween 80, Cholesterol (CHOL) และ polyethylene glycol (PEG) 400 โดยแต่ละสูตรมีอัตราส่วนและความเข้มข้นทั้งหมดของไขมันแตกต่างกัน และทำการศึกษาลักษณะทางกายภาพ, คุณสมบัติของนาโนโซมในด้านขนาดอนุภาค โดยใช้ Photon Correlation Spectroscopy และ % entrapment efficiency ด้วยวิธี DPPH radical scavenging assay, ศึกษาความคงสภาพของนาโนโซมโดยกระบวนการ Freeze-Thaw เพื่อประเมินถึงขนาดอนุภาคและ entrapment efficiency และศึกษาการซึมผ่านผิวหนังสัตว์ทดลอง โดยใช้ modified Franz's diffusion cell จากการศึกษาพบว่า สูตรนาโนโซมของน้ำมันรำข้าวที่เหมาะสม คือ SPC:Tween 80 (84:16 weight ratio), SPC:CHOL:PEG 400 (7:2:1 molar ratio) ที่ความเข้มข้นของไขมันระหว่าง 30-50 μmol ซึ่งจากการประเมินคุณสมบัติของนาโนโซม พบว่า แต่ละสูตรมีขนาดและความสามารถในการกักเก็บสารสำคัญที่

เหมาะสม และเมื่อประเมินความสามารถในการซึมผ่านผิวหนัง โดยการเปรียบเทียบระหว่างน้ำมันรำข้าวที่อยู่ในรูปแบบนาโนโซม พบว่านาโนโซมทั้ง 3 สูตร สามารถซึมผ่านผิวหนังได้ดีกว่าน้ำมันรำข้าวอย่างมีนัยสำคัญ โดยตำรับ SPC:CHOL:PEG 400 (7:2:1) ที่ความเข้มข้นของไขมัน เท่ากับ 30 μmol สามารถซึมผ่านผิวหนังได้ดีที่สุด เพื่อประเมินศักยภาพของการนำนาโนโซมของน้ำมันรำข้าวมาใช้เป็นสารต้านอนุมูลอิสระในสูตรตำรับ ได้เตรียมครีมซึ่งมีนาโนโซมของน้ำมันรำข้าวในความเข้มข้นร้อยละ 5, 10 และ 20 โดยน้ำหนักเป็นสารสำคัญ และได้เตรียมตำรับครีมซึ่งมีน้ำมันรำข้าวเป็นสารสำคัญที่ความเข้มข้นเดียวกัน พบว่าทุกตำรับของนาโนโซมของน้ำมันรำข้าวมีลักษณะทางกายภาพและความสามารถในการต้านอนุมูลอิสระดีกว่าตำรับของน้ำมันรำข้าวหลังจากการผ่านการทดสอบความคงตัวของตำรับในสภาวะเร่ง และเมื่อประเมินความสามารถในการซึมผ่านผิวหนัง พบว่าทุกตำรับของนาโนโซมของน้ำมันรำข้าวสามารถซึมผ่านผิวหนังได้ดีกว่าตำรับของน้ำมันรำข้าวอย่างมีนัยสำคัญ โดยตำรับ SPC:CHOL:PEG 400 (7:2:1) ที่ความเข้มข้นของไขมัน เท่ากับ 30 μmol สามารถซึมผ่านผิวหนังได้ดีที่สุด จึงสรุปได้ว่าตำรับน้ำมันรำข้าวในรูปของนาโนโซมมีศักยภาพในการนำมาใช้เป็นสารต้านอนุมูลอิสระในทางเครื่องสำอาง

Thesis Title Development of Rice Bran Oil Nanosome for Using in Cosmetic

Author Miss Pariporn Maharachatahtakul

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ABSTRACT

Rice bran is a component of raw rice obtained from rice milling process. Rice bran oil contains many different antioxidants such as gamma – oryzanol , tocopherols and tocotrienols. Using nano-technology in preparing rice bran oil nanosome will improve its ability in skin permeation. The objective of the present investigation was to develop nanosome of rice bran oil for using in cosmetic skin care products. The antioxidant activity of rice bran oil was evaluated by DPPH radical scavenging assay, the obtained solution exhibited antioxidant activity with EC_{50} value of 4.396 ± 0.282 mg/ml. Furthermore, the development of nanosomes of rice bran oil in order to improve its skin permeation ability were also studied. Rice bran oil nanosomes were prepared by reverse phase evaporation method using soybean phosphatidyl choline (SPC), Tween 80, Cholesterol (CHOL) and polyethylene glycol (PEG) 400 in various ratios and total lipid contents. The nanosomes were evaluated for their particle sizes by Photon Correlation Spectroscopy and entrapment efficiency by

antioxidant evaluation using the DPPH radical scavenging assay. The stability of nanosomes under freeze-thaw condition was evaluated in terms of size and entrapment efficiency. The *in vitro* skin permeation of nanosomes, in term of antioxidant activity, was also determined using modified Franz's diffusion cell. The suitable formulations include SPC:Tween 80 (84:16 weight ratio), SPC:CHOL:PEG 400 (7:2:1 molar ratio) with the total lipid of 30 to 50 μmol. All formulations show appropriate properties in both size and entrapment efficiency. From *in vitro* animal skin permeation studies, all nanosome formulations provided significantly higher permeation than that of pure rice bran oil. The highest skin permeation was observed in nanosome with SPC:CHOL:PEG 400 (7:2:1) total lipid 30 μmol. In order to evaluate the rice bran oil nanosome as an antioxidant in skin care formulation, creams containing 5 %, 10 % and 20 % w/w of rice bran oil nanosome and rice bran oil creams with the same concentration were prepared. All preparations of rice bran oil nanosome showed good stability and higher antioxidant efficiency than those of rice bran oil. The *in vitro* skin permeation results show that all nanosome formulations gave significantly higher permeation than that of rice bran oil cream. The nanosome cream formulation containing SPC:CHOL:PEG 400 (7:2:1) with total lipid 30 μmol showed the best skin permeability. It can be concluded that rice bran oil nanosome has a potential to be used as an antioxidant in cosmetic formulations.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	absorbance
α-	alpha-
β-	beta-
BHT	butylated hydroxytoluene
BHA	butylated hydroxyanisole
BRBO	the concentrated bioactive components from rice bran oil
^{o}C	degree Celsius
cm	cetimeter
CHOL	cholesterol
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	effective concentration of sample requires scavenging free radical by 50%
EIV	ether injection vesicle
FPV	French press vesicle
EPC	phosphatidylcholine from dried egg yolk
g	gram
G-6-PD	glucose-6-phosphate dehydrogenase
GSHP	glutathione peroxidase
hr	hour

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

HDL	high-density lipoprotein
kg	kilogram
L	liter
LDL	low-density lipoprotein
LPO	lipid peroxidation
LUV	large unilamellar vesicle
μg	microgram
μl	microliter
mg	miligram
ml	milliliter
mM	millimolar
M	molar
MLV	multilamellar vesicle
nm	nanometer
PCS	photon correlation spectroscopy
PEG	polyethylene glycol
rpm	round per minutes
REV	reverse phase evaporation vesicle
ROS	reactive oxygen species
SA	stearylamine
SD	standard deviation

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

SPC	phosphatidylcholine from soybean
SOD	superoxide dismutases
SUV	small unilamellar vesicle
ULV	unilamellar vesicle
UV	ultraviolet
UVA	ultraviolet A
UVB	ultraviolet B

CHAPTER 1

INTRODUCTION

1.1 General introduction

In recent years, there has been increasing evidence that free radicals are associated with pathological conditions such as atherosclerosis, carcinogenesis and aging. Basically, free radicals and other reactive oxygen species including hydroxyl radical, superoxide radical and other singlet oxygen are continually formed in the human body since they are by products of a variety of pathways in aerobic metabolism. In addition, free radicals derived from the environment, especially ultraviolet radiation are important extrinsic factors accelerating aging (Ames *et al*., 1993; Rieger, 1993; Halliwell and Gutteridge, 1989; Wickens, 2001 and Bokov *et al*., 2004). They also cause damage to connective tissue components of the dermis, particularly collagen, leading to premature skin age and deepening wrinkles (Jenkins, 2002). Generally, the defense mechanisms of the skin against oxidative damage need antioxidant compounds such as ascorbic acid, tocopherols, selenium and antioxidant enzymes such as glutathione peroxidase and glutathione reductase. Such compounds can, therefore, diminish with aging.

Nowadays, synthetic phenolic compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), are used as antioxidants in fat–containing formulations; however, their harmlessness is at present a controversial point (Ito *et al*., 1986; Whysner *et al*., 1994 and Williams *et al*., 1999), therefore there has been a global trend toward the use

of natural substances present in fruits, vegetables, oilseeds, cereal grain and herbs as antioxidants and functional ingredients (Farr, 1997; Wang *et al*., 1997 and Son *et al*., 2003). Several of these substances are believed to play a potential role to interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen in food and biological systems (Shahidi and Wanasundara, 1992; Sánchez-Moreno *et al*., 1999). For example, some vitamins and their derivatives have important biological roles related to the inhibition of lipid peroxidation and free radical scavenging in the human body as well as controlling the occurrence of lipid oxidation in food systems (Servinova *et al*., 1991).

Rice bran is a component of raw rice that is obtained when it is removed from the starchy endosperm in the rice milling process (Lakkakula *et al*., 2004). Although, it has been recognized as an excellent source of vitamins and minerals, it has been utilized as a human food and has traditionally been used primarily in animal foods. Research conducted in the last two decades has shown that it contains a unique complex of naturally occurring antioxidant compounds (Moldenhauer *et al*., 2003). Current research has shown that rice bran may contain even 100 different antioxidants, as new ones are being discovered (Bidlack., 1999). Among the most powerful of these are tocopherols, tocotrienols and gamma–oryzanol (Shin *et al*., 1997; Xu and Godber, 1999). Antioxidant compounds in rice bran oil have professed health benefits as well as their antioxidant characteristics for improving the storage stability of foods. Also, it has been determined that the amount of cholesterol lowering occurs to a greater extent than expected from the fatty acid composition of oil,

suggesting that besides fatty acids, other components in the oil were responsible for the cholesterol lowering effect (Rogers *et al*., 1993).

Because of good properties of rice bran oil, using nano-technology in preparing rice bran oil liposome will improve its ability in skin permeation and increase efficacy in product and increase the value of rice from normal agricultural goods to be cosmetic product. It can be used safety as antioxidant instead of BHA and BHT in cosmetic formulation in pharmaceutical applications according to its natural origin.

The aims of the present study were to follow

1. Develop rice bran oil nanosome formulations and determine their antioxidant activity using the DPPH radical scavenging assay
2. Determine the physicochemical stability of rice bran oil nanosome
3. Study antioxidant activity of rice bran oil nanosome formulations *in vitro* skin penetrated by the DPPH radical scavenging assay
4. Prepare creams containing rice bran oil nanosome and evaluate their physical stability and their antioxidant activity *in vitro* skin penetrated

1.2 Literature review

1.2.1 Free radicals and skin aging

The idea that free radicals can cause aging was offered by Denham Herman in 1956. He proposed that aging and age-related disease might be due to the long term effect of oxidative damage by free radicals which, in turn, are modified by genetic and environmental factors (Wickens, 2001).

According to the free radical theory of aging, cellular senescence is a cumulative oxidative damage by free radicals, a causative factor in aging. Skin is constantly exposed to various environmental insults such as exposure to UV and ionizing radiation, oxygen, ozone and pollutants that may deleteriously augment the normally occurring intracellular oxidative stress. Skin is also a major candidate and target of oxidative damage by free radicals. Lipids, proteins and DNA are biological sites of the skin which are candidates for oxidative damage (Kohen, 1999). The signs of skin aging are commonly associated with increased wrinkling, sagging and increased laxity (Jenkin, 2002).

Free radicals are normal biochemical intermediates of any metabolic reactions. They consist of any chemical species (atom, ions or molecules) that contain one or more unpaired electrons in their outer atomic or molecular orbital. This makes them highly unstable and violently reactive (Benedetto, 1998 and Wickens, 2001). The most important free radicals found are reactive oxygen species (ROS) which include oxygen free radicals or oxygen-centered free radicals and non radical species as illustrated in Table 1.1.

ROS are one of the major and important contributions to skin aging, skin disorders and skin diseases (Benedetto, 1998 and Kohen, 1999). They are mostly formed in mitochondria where oxygen is reduced in four sequential steps to produce water. This chain reaction produces a number of short-lived intermediates including surperoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) (Ames et al., 1993) In addition, the intracellular formation of these free radicals can be stimulated by the environmental sources, especially UVA and UVB radiation (Halliwell and Gutteridge, 1989). H_2O_2 may intiate a peroxidation process in either lipids or proteins. This lipid peroxidation process may lead to a change in the fluidity of the plasma membranes resulting in a molecule leakage and a subsequent dysfunction in its essential roles. In addition, ROS may also directly inactivate enzymes and cause protein and DNA degradation. Damage to DNA may result in deleterious process, aging, as well as onset of cancer and other pathological disorders (Kohen, 1999 and Vendemiale et al., 1999).

Table 1.1 Reactive oxygen species (ROS)

Oxygen radical		Non-radical oxygen	
Superoxide anion	$O_2^{\cdot-}$	Hydrogen peroxide	H_2O_2
Hydroxyl	$OH^{\cdot}$	Hypochlorous acid	HOCl
Peroxyl	$ROO^{\cdot}$	Ozone	O_3
Alkoxyl	$RO^{\cdot}$	Singlet oxygen	
Hydroperoxyl	$HOO^{\cdot}$		

1.2.2 Defense mechanism of skin aginst oxidative damage

The epidermis of the skin possesses an extremely efficient antioxidant activity that is superior to most tissues (Jenkins, 2002). There are two types of antioxidants, the enzymatic and non enzymatic antioxidants (Benedetto, 1998).

The enzymatic antioxidants including superoxide dismutases (SOD), catalase, glutathione peroxidase (GSHP) and glucose-6-phosphate dehydrogenase (G-6-PD), protect cells by hastening biochemical reaction. Moreover, thioredoxin reductase, catalase and GSHP/reductase are the main antioxidant enzymes which are involved in the protection of the epidermis against UV-radiation-generated ROS (Benedetto, 1998).

The nonenzymatic antioxidants, including ascorbic acid (vitamin C), α-tocopherol (vitamin E), retinol (vitamin A), β-carotene and glutathione, help dissipate intracellular oxidants or ROS by acting as free radical scavengers, thereby maintaining intracellular redox homeostasis and reducing the potential for cellular oxidative damage (Benedetto, 1998). However, UV radiation exposure (both acute and chronic) causes a decrease of these nonenzymatic antioxidants in cell diminishing their role as oxidant quenchers and free radical scavengers. Hence, the rationale for the suggestion that vitamin A, C, E and their derivatives might be effective in the prevention of skin damage caused by ROS that are induced by UV radiation (Benedetto, 1998).

1.2.3 Rice bran oil

Rice is one of the widely used crops in the world for human consumption. More than 500 million metric tons of rice is produced per year. Cultivated rice (Oryza sativa L.), which belongs to the Gramineae or grass family (Genus Oryza), has been consumed by humans for almost 5000 years. It has adapted to diverse environments and currently sustains two–thirds of the world' s population (International Rice Commission, 1998). Paddy rice is milled to separate the rice kernel from the hull and bran. Rice bran contains about 18-22 % rice bran oil. Rice bran is rich in vitamins, minerals, amino acids, essential fatty acids and antioxidant nutrients. Rice bran is used by the food industry in the production of baked goods, snacks, crackers, breads and cereals. Rice bran oil has an impressive nutritional quality, which makes it suitable for nutraceutical production. Nutraceuticals are defined as "a food or naturally occurring food supplement thought to have a beneficial effect on human health".

Crude rice bran oil contains high levels of components with Antioxidant properties: tocopherols/tocotrienols and gamma–oryzanol (Shin *et al*., 1997; Xu and Godber, 1999). Initially, gamma–oryzanol was thought to be a single compound, but now it is known that it is a mixture of at least 10 phytosteryl ferulates; cycloartenyl ferulate, 24–methylenecycloartanyl ferulate and campesteryl ferulate have been identified as the major components, accounting for 80% of gamma–oryzanol in rice bran oil (Xu and Godber, 1999).

Figure 1.1 : Rice (scientific name : *Oryza sativa*)

Gamma-oryzanol is a naturally occurring mixture of plant chemicals called sterols and ferulic acid esters. Although it appears in grains such as barley and corn, gamma-oryzanol is extracted from rice bran oil for commercial purposes. Gamma-oryzanol is actually two molecules in one. The largest part is the triterpenyl alcohol part. This is simply another name for sterol. Sterols are the group of compounds found throughout nature, with many vital biological functions. Some well-known sterols are cholesterol and beta sitosterol. Many hormones such as testosterone, estrogen, progesterone, and corticosteroids, are derived from cholesterol, and can be thought of as modified sterols. The second half of each gamma-oryzanol molecule is ferulic acid, a widespread plant compound.

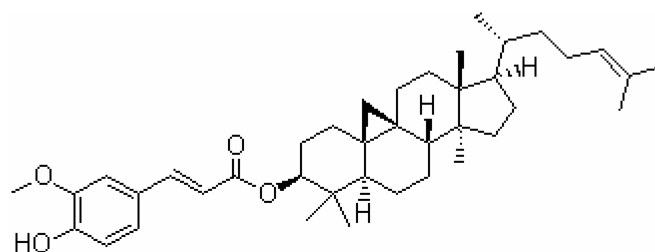

Figure 1.2: Chemical structure of gamma-oryzanol (Chemblink, 2007)

Table 1.2: Chemical characteristics of gamma-oryzanol (Chemblink, 2007)

Characteristics	**Value**
Molecular Formula	$C_{40}H_{58}O_4$
Molecular Weight	602.89
Melting point	135-137°C

Gamma-oryzanol/ferulic acid is a potent membrane antioxidant in animals and humans. The role of antioxidants in exercise physiology has only recently been explored and elucidated. Several reviews have illustrated the mechanism by which anaerobic exercise actually produces free radicals, which result in fatigue. Surprisingly, the increased flow of oxygen to fuel working muscles is not a major source for free radical damage, as normal cellular antioxidant levels seem to be adequate for the amount of free radicals produced by oxidative processes. Instead, free radicals that overwhelm cellular defenses are produced by the action of xanthine oxidase on inosine in muscle tissue. (Greatvista chemicals, 2007).

Gamma–oryzanol has been reported to possess some health beneficial properties especially antioxidant:

Juliano *et al*. (2005) studied the molecular mechanism (s) of the antioxidant activity of gamma–oryzanol by utilizing different in vitro model systems, such as scavenging of stable 2,2-diphenyl-1-pic-rylhydrazyl radical ($DPPH^{\cdot}$), $OH^{\cdot}$ and $O_2^{\cdot -}$ radicals scavenging, and azo compound AMVN–initiated lipid peroxidation. The effect of gamma–oryzanol on the oxidative stability of vegetable oils of pharmaceutical and cosmetic interest was then evaluated in an oxidation accelerated oxidation test and compared with the effect of the well–known antioxidants BHA and BHT. The gamma–oryzanol is an organic radical scavenger capable of preventing AMVN–triggered lipoperoxidation. Moreover, the free radical scavenging action of gamma–oryzanol and their protection effect against lipoperoxidation make it a good candidate for use as natural antioxidant of technological interest. To verify its applicability in protection of lipidic raw materials, different oil samples were added with increasing concentrations of gamma–oryzanol and then subjected to an accelerated oxidation test. Gamma–oryzanol produced a dose–dependent increase of the induction time with a maximum effect at 10 mmol/kg and an efficiency comparable to that one of BHT. Its protective effect from lipoperoxidation induced by heating and oxygen exposition was especially interesting in oils rich in polyunsaturated fatty acids, such as rosa mosqueta oil and grape seed oil.

Ha *et al*. (2005) studied the effects of bioactive substances in rice bran oil on lipid levels and lipid peroxidation in serum and liver. Three groups of male Sprague–Dawley rats were fed following experimental diets : normal diet, high-cholesterol diet and high-cholesterol diet supplemented with the concentrated bioactive components from rice bran oil (BRBO) for four weeks. The liver cholesterol and triacylglycerol contents were higher in rats fed with the high–cholesterol diet than those fed with the normal diet but significantly decreased by BRBO supplementation. Similarly, hepatic thiobarbituric acid–reactive substances were increased by a high–cholesterol diet and reduced by BRBO supplementation in rats. The high–cholesterol diet group but it was significantly increased in rats of the BRBO group. In addition, BRBO recovered the activities of serum aspartate aminotransferase which was elevated in rats by a high–cholesterol diet.

Xu *et al*. (2001) studied the antioxidant activities of vitamin E (alpha-tocopherol, alpha-tocotrienol, gamma–tocopherol and gamma–tocotrienol) and gamma-oryzanol components (cycloartenyl ferulate, 24-methylenecycloartanyl ferulate and campesteryl ferulate) purified from rice bran in a cholesterol oxidation system accelerated by 2,2´-azobis (2–methylpropionamidine) dihydrochloride. All components exhibited significant antioxidant activity in the inhibition of cholesterol oxidation. The highest antioxidant activity was found for 24-methylenecycloartanyl ferulate, and all three gamma-oryzanol components had activities higher than that of any of the four vitamin E components. Because the quantity of gamma-oryzanol is up to 10 times higher than that of vitamin E in rice bran, gamma-oryzanol may be a more important antioxidant

of rice bran in the reduction of cholesterol oxidation than vitamin E, which has been considered to be the major antioxidant in rice bran. The antioxidant function of these components against cholesterol oxidation may contribute to the potential hypocholesterolemic property of rice bran.

Cicero and Gaddi (2001) studied the rice bran oil and gamma–oryzanol in the treatment of hyperlipoproteinemias and other conditions. Diet is the first (and sometimes the only) therapeutic approach to hyperlipoproteinaemias. Rice bran oil and its main components (unsaturated fatty acids, triterpene alcohols, phytosterols, tocotrienols, alpha-tocopherol) have demonstrated an ability to improve the plasma lipid pattern of rodents, rabbits, non- human primates and humans, and thereby reducing total plasma cholesterol and triglyceride concentration and increasing the high density lipoprotein cholesterol level. Other potential properties of rice bran oil and gamma–oryzanol, which comprised modulation of pituitary secretion, inhibition of gastric acid secretion, antioxidant action and inhibition of platelet aggregation, were also studied both *in vitro* and in animal models.

Yoshino *et al.* (1989) studied the hypercholesterolemic effect of gamma-oryzanol which was investigated in 67 patients with hyperlipidemia. Three milligrams of gamma– oryzanol were administered daily for 3 months. Plasma cholesterol and plasma triglyceride levels decreased significantly from the second month and after three months, respectively. High–density lipoprotein (HDL)–cholesterol was also significantly elevated after three months. The reduction in plasma–cholesterol was attributable to the decrease in low–density lipoprotein (LDL) cholesterol.

Moreover, gamma-oryzanol has been reported to be used in the cure of nerve imbalance, disorders of menopause (Rogers *et al*., 1993) and used to decrease of hepatic cholesterol biosynthesis and plasma cholesterol (Rong *et al*., 1997) , it was also used as an antioxidant in *in vitro* systems, such as pyrogallol autoxidation (Kim *et al*., 1995), lipid peroxidation induced in porcine retinal homogenate by ferric ion (Hiramitsu and Armstrong, 1991); furthermore, it has been proposed as a UV-A filter in sunscreen cosmetics. It seems reasonable to assume that gamma-oryzanol can also be used as antioxidant for pharmaceutical purposes.

1.2.4 Liposome

Liposomes are submicron vesicles of which structure is closed by one or more concentric of an amphiphile molecules (usually phospholipids) surrounding an aqueous compartment (Figure 1.3). A liposome population of vesicles mat range in size from ten nanometers to ten microns in diameter (New, 1990). The structure of the liposome bilayer is similar to cellular membranes. Similar to the structural arrangement found in living cells, phospholipids are the major components of the lipid bilayer. The phospholipids spontaneously form bilayers and liposomes in aqueous systems because of their amphipathic character. The hydrophobic region, the fatty acid portion, is shielded from the water by facing the inside of the lipid bilayer and the hydrophilic region, the polar head group consisting of phosphoric acid and an alcohol, is immersed in the aqueous environment by facing the outside of the lipid bilayer (Patent Storm, 2007).

Liposomes were discovered in the 1960s (Bangham *et al.*, 1963). Since then, they were utilized as model membranes to study transport of molecules across bilayers, lipid-protein interactions and physicochemical properties of amphiphatic molecules. Liposomes are potential drug carriers for a variety of drugs. They entrap a quantities of materials both within their aqueous compartment (for hydrophilic molecules) and within the membrane (for hydrophobic molecules). Therefore, liposmes have attracted considerable attention use in drug delivery application (New, 1990; Swarbrick and Boyland, 1994; Verumi and Rhodes, 1995; Malmsten, 2002).

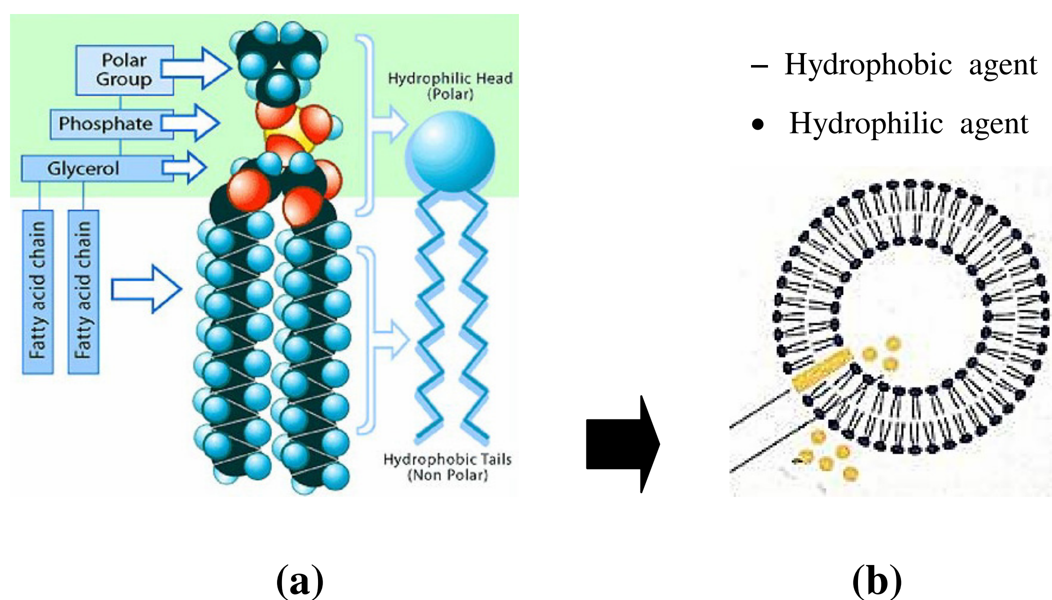

Figure 1.3 Structure of (a) phospholipid molecule (Azaya Therapeutics, 2008) and (b) liposome vesicle (HBC Protocol Inc., 2008)

1.2.4.1 Classification of liposome

There are various classes of liposomes. Liposomes are classified either by the method of their preparation or by the number of bilayers present in the vesicle, or by their size as shown in Table 1.3. The description of liposomes by the lamellarity and size are common than by the method of their preparation (Verumi and Rhodes, 1995).

Table 1.3 Nomenclature and approximate sizes of various liposomes (Verumi and Rhodes, 1995)

Liposome	Classification	Approximate size (nm)
By size	Small unilamellar vesicle (SUV)	25-50
	Large unilamellar vesicle (LUV)	100
By lamellarity	Multilamellar vesicle (MLV)	5-10000
	Unilamellar vesicle (ULV)	25-100
By method	Reverse phase evaporation vesicle (REV)	500
	French press vesicle (FPV)	50
	Ether injection vesicle (EIV)	20

Mezei and Gulasekharam (1980, 1982), Singh and Mezei (1983) and Schaeffer and Krohn (1982) were the first to report the potential use of liposomes in topical applications for the skin and eyes. Many studies performed in the last decade showed significantly higher absorption rates (Michel *et al*., 1992; Cevc, 1996), as well as greater pharmacological effects for drugs applied to the skin entrapped in liposomes, as compared to conventional

topical formulations (Skalko *et al*., 1992; Sharma *et al*., 1994). Two in vivo rabbit studies document comparisons between liposomal and conventional formulations of triamcinolone acetonide (Mezei and Gulasekharam, 1980, 1982). In both studies, the application of the liposomal preparations was associated with greater steroid concentrations in the epidermis and dermis and a lower systemic absorption than the regular formulations. Further, biodeposition studies in animals have demonstrated that liposomal encapsulation can improve the penetration of various molecules.

It is reported by several authors that the high elasticity of vesicles could result in enhanced drug transport across the skin as compared to vesicles with rigid membranes (Planas *et al*., 1992; Sentjurc and Gabrijelcic, 1995; Cevc *et al*., 1998; Paul *et al*., 1998; van den Bergh *et al*., 1999; Guo *et al*., 2000a,b). It seems that liposomes with a heterogeneous lipid composition, or in other words, with several coexisting domains exhibiting different fluidity characteristics in the bilayer (Vrhovnik *et al*., 1998) can be used to enhance the penetration of entrapped drugs into the skin. It is supposed that once in contact with skin, some budding of liposomal membrane might occur (Cevc *et al*., 1995; Vrhovnik *et al*., 1998). This could cause a mixing of the liposome bilayer with intracellular lipids in the SC (Egbaria *et al*., 1991), which may change the hydration conditions and thereby the structure of lipid lamellae. This may enhance the permeation of the lipophilic drug into the SC and ease the diffusion of hydrophilic drugs into the interlamellar spaces. On the other hand, it may be possible that some vesicles, which are deformable enough, will pass the SC as intact structure (Cevc and Blume, 1992; Cevc *et al*., 2002) or may

accumulate in the channel-like regions in the SC (Honeywell-Nguyen *et al*., 2000) depending upon their compositions. Mode of application of the vesicles has been another issue of discussion initially addressed by Cevc and co-workers in 1992. They reported that the flexible vesicles work more efficiently under non-occlusive application. Non-occlusive application is the key to create a transepidermal osmotic gradient, which is believed to be the driving force for the transport of vesicles into the skin (Cevc and Blume, 1992).

1.2.5 Nanosome

Nanosome is a microscopic particle with at least one dimension less than 100 nm. Nanosomes are comprised of a single phospholipid bilayer. Nanosomes are not formed spontaneously; they are created by down–sizing large liposomes with ultrasonic energy. Nanosomes are interactive liposomes, making them not only a powerful delivery system of beneficial nutrients to the skin, but also a powerful removal system for oily discards. Via the cell membrane, they can deliver many molecules to cells, including the cells' own building blocks, the phospholipids. They can also remove from cells various molecules via their membranes, including phospholipids and cholesterol. Nanosomes can also exchange molecules with cell membranes, giving them the molecules they need and taking from them molecules they should discard. Nanosomes carry vital ingredients such as anabolites, nutrients, antioxidants, and cell stimulants including vitamins C, A, E, B and others, into the skin. Nanosomes can amplify the effectiveness of their entrapped ingredients they carry (Elsom research, 2007).

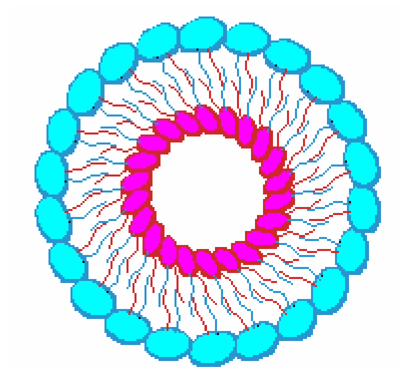

Figure 1.4: General Structure of a Nanosome (Elsom research, 2007)

Interest in the use of nanosomes as drug carriers in the field of dermatological treatment has been increasing during recent years. In animal experiments the nanosomal form, compared to the conventional dosage forms (ointment, cream, gel, lotion), provided higher drug concentration in the intended site of action i.e. the skin, and gave the lower concentration in the internal organs, which were the possible site of adverse or unwanted effects.

Therefore, the aims of the present study are to prepare rice bran oil nanosome for using in cosmetic and evaluate the effectiveness of antioxidant activity of nanosome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Rice bran oil was supplied by Thai Edible Oil Co., Ltd (Thailand). Egg yolk phosphatidylcholine (EPC), soybean phosphatidylcholine (SPC) and Cholesterol (CHOL) were purchased from Fluka Biochemika Company.

2.1.2 Chemicals and reagents

2.1.2.1 Antioxidant assay

Absolute Ethanol, AR grade (Merck®, Darmstadt, Germany)

Chroloform (Merck®, Darmstadt, Germany)

Ethanol 95 % (Merck®, Darmstadt, Germany)

Ether (Merck®, Darmstadt, Germany)

1,1-diphenyl-2-picrylhydrazyl (Sigma®, St.Louis, USA)

Polyethylene glycol 400, PEG 400 (Srichand Co., Ltd., BKK, Thailand)

Polyethylene glycol 4000, PEG 4000 (Srichand Co., Ltd., BKK, Thailand)

Stearyl amine (Aldrich®, Steinheim, Germany)

(±)-α-Tocopherol, AR grade (Fluka®, Buchs, Switzerland)

Triton x 10% (J.T. Baker, Philipsburg, USA)

2.1.2.2 Formulation development

Cetearyl octanoate, Lanol 1688® (ADINOP Co., Ltd., BKK, Thailand)

Ceteareth-20, Polyoxyethylene-20-Cetystearyl alcohol, Cetomacrogol 1000 (BASF, Ludwigshafen, USA)

Cetostearyl alcohol (Srichand Co., Ltd., BKK, Thailand)

Cetyl alcohol (Vidhyasom, BKK, Thailand)

Ceteareth-25, Cremophor A 25 (BASF, Ludwigshafen, USA)

Ceteareth-6-(and) Stearyl alcohol, Cremophor A6 (BASF, Ludwigshafen, USA)

Glyceryl monostearate, SE (Vidhyasom, BKK, Thailand)

Glycerin (Vidhyasom, BKK, Thailand)

Hard paraffin (Srichand Co., Ltd., BKK, Thailand)

Isopropyl myristate (Vidhyasom, BKK, Thailand)

Mineral oil (Vidhyasom, BKK, Thailand)

Polyacrylamide / c 13, 14 Isoparaffin / Laureth–7–Seppic, Sepigel 305® (ADINOP Co., Ltd., BKK, Thailand)

Polyoxyethylene (20) sorbitan monooleate, Tween 80® (Srichand Co., Ltd., BKK, Thailand)

Stearic acid (Srichand Co., Ltd., BKK, Thailand)

Sorbitan monooleate, Span 80® (Srichand Co., Ltd., BKK, Thailand)

Phenoxyethanol, Methyl-, Ethyl-, Propyl-, Butylparaben, Uniphen 0.5% (Namsiang International Co., Ltd, BKK, Thailand)

2.2 Instruments

Photon Correlation Spectroscopy (PCS) or Laser light scattering spectrophotometer (Malvern instruments) Mastersizer (Malvern , UK).

Microplate reader (PowerWave X, Bio–TEK Instruments Inc.)

Water bath (WB-14, Memmert, Germany)

Hot air oven (DIN 12880-KI, Memmert, Germany)

UV–visible spectrophotometer (Genesys ™ 5, Spectronic Instruments, USA).

Magnetic stirrer (Heidolph, MR 3000D, Germany)

Sonicator (Elma ®, Transsonic 310/H)

Vortex (G–560E, Scientific, USA)

pH meter (Orion, Model 410A, USA)

Rotary evaporator (Eyela, N-1000 series, Tokyo Rikakikai Co.,Ltd.)

Balance digital Scale (Sartorius, type 1712, Gottingen, Germany)

Centrifuge (Hermle®, model Z323K, Germany)

Modified Franz diffusion cell (Hanson®, model 57-6M, USA)

Multiple stirrer (IKA®-Werke, model RO5 Power, Germany)

Optical Microscophy (Olympus®, model CK2, Japan)

Viscometer (Brookfieled dial reading, Model RVT, USA)

2.3 Methods

2.3.1 Assay for antioxidant activity

The antioxidant activity of rice bran oil with different oryzanol content (high and low oryzanol) was evaluated by DPPH radical scavenging assay which was originally described by Blois (1958). The solvent used for dissolved the oil was ethanol:ether (1:2).

2.3.1.1 DPPH radical scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable free radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as deep violet color and shows a strong absorption band at 520 nm. The DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule, at which the absorption vanishes and the resulting decolorization is stomachiometric with the number of electrons taken up; the solution has pale violet color (Blois, 1958). A DPPH solution having a concentration of 6×10^{-5} M was used in the present study since at this low concentration the color is not too dense and the Lambert-Beer law is obeyed. If the tested substance is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has an antioxidant effect by the mechanism of free radical scavenging activity. The following assay procedure was modified from those described by Yamasaki *et al*., (1994).

2.3.1.2 Preparation, testing procedure and data processing

A test sample was dissolved in an organic solvent (ether:ethanol, 2:1) to obtain a stock solution (1 mg/ml) and then diluted for at least four dilutions (100, 50, 10 and 1 mg/ml). Each concentration was tested in triplicate. A portion of sample solution (0.5 ml) was mixed to an equal volume of 6×10^{-5} M DPPH solution (in absolute ethanol). After 30 minutes at room temperature, the mixture was measured with an absorbance (A) of 520 nm by UV spectrophotometer. The standard, control and blank solutions in each experiment are as follows :

Control : 500 µl of solvent + 500 µl of $6x10^{-5}$ M of DPPH in absolute ethanol

Control blank : 500 µl of solvent + 500 µl of absolute ethanol

Sample : 500 µl of sample + 500 µl of $6x10^{-5}$ M of DPPH in absolute ethanol

Sample blank : 500 µl of sample + 500 µl of absolute ethanol

Vitamin E was utilized as a standard compound or positive control for this assay. The free radical scavenging activity of each sample was determined corresponding to the intensity of quenching DPPH. The result was expressed as the percentage inhibition calculated as the following equation:

$$\% \text{ inhibition} = \left[\frac{(A_{control} - A_{sample})}{A_{control}}\right] * 100$$

Where $A_{control}$: absorbance of DPPH solution without sample solution

A_{sample} : absorbance of DPPH solution with sample solution

The results were calculated in term of radical scavenging activity (EC_{50}) which reflex antioxidant activity of the sample. The value of EC_{50} (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations of samples.

2.3.2 Preparation of Nanosomes

Nanosomes of rice bran oil were prepared by reversed phase evaporation method. The composition of nanosomes were optimized in terms of type and ratio of both lipids and additives used as shown in Table 2.1.

Ingredient used :

Lipid	:	SPC (Phosphatidylcholine from soybean)
		EPC (Phosphatidylcholine from dried egg yolk)
Additive	:	Cholesterol (CHOL)
		Tween 80

Surface charge : Stearyl amine (SA)

Polyethylene glycol 400 (PEG 400)

Polyethylene glycol 4000 (PEG 4000)

Ratio of nanosome composition :

Lipid : CHOL : SA (or PEG 400, PEG 4000) = 7:2:1 (molar ratio)

Lipid : Tween 80 = 84 : 16 (weight ratio)

Phospholipid and an additive with a 7:2:1 molar ratio (or 84:16 weight ratio) were dissolved in 6 ml of ethanol. Rice bran oil was dissolved in a solvent mixture (6 ml) of ether and ethanol (2:1 v/v). In each formulation, the concentration of rice bran oil was 25 mg/ml, and the total lipid was varied from 20 to 50 µmol. Rice bran oil was mixed in lipid solution and heated to 30°C in rotary evaporatory. Then, the organic phase (ether) was evaporated from the sample connected to a vacuum pump (Millipore®). The water bath was mantained at 60°C. After that, 6 ml of water was added into the mixture at 60°C, and the mixture was swirled continuously for 1 minutes. The ethanol was evaporated by 60°C. The obtained nanosome suspension (6 ml) was then further analysed.

Table 2.1 Formualtion of rice bran oil nanosomes

Composition	Molar ratio	Total lipid
SPC : CHOL : SA	7 : 2 : 1	20
EPC : CHOL : SA	7 : 2 : 1	20
SPC : CHOL : PEG 400	7 : 2 : 1	20
EPC : CHOL : PEG 400	7 : 2 : 1	20
SPC : CHOL : PEG 4000	7 : 2 : 1	20
EPC : CHOL : PEG 4000	7 : 2 : 1	20
SPC : CHOL : SA	7 : 2 : 1	30
EPC : CHOL : SA	7 : 2 : 1	30
SPC : CHOL : PEG 400	7 : 2 : 1	30
EPC : CHOL : PEG 400	7 : 2 : 1	30
SPC : CHOL : PEG 4000	7 : 2 : 1	30
EPC : CHOL : PEG 4000	7 : 2 : 1	30
SPC : CHOL : SA	7 : 2 : 1	50
EPC : CHOL : SA	7 : 2 : 1	50
SPC : CHOL : PEG 400	7 : 2 : 1	50
EPC : CHOL : PEG 400	7 : 2 : 1	50
SPC : CHOL : PEG 4000	7 : 2 : 1	50
EPC : CHOL : PEG 4000	7 : 2 : 1	50
Composition	**Weight ratio**	**Total lipid**
SPC : Tween 80	84 : 16	20
EPC : Tween 80	84 : 16	20
SPC : Tween 80	84 : 16	30
EPC : Tween 80	84 : 16	30
SPC : Tween 80	84 : 16	50
EPC : Tween 80	84 : 16	50

2.3.3 Physical characteristic of rice bran oil nanosome

2.3.3.1 The particle size of nanosomes were determined using the Photon Correlation Spectroscopy with an Autosizer Lo-C (Malvern Instruments, Worcestershire, UK) at 25 ^{o}C with a fixed angle of 90 degrees. The measurement was done in triplicate for each sample. The nanosome suspensions (200 µl) were diluted with 4 ml filtered de-ionized water and shaken manually about 0.5 min for complete hydration. Nanosome suspensions were analyzed at 25°C. A size distribution analysis of the data was done according to the instrument manual.

2.3.3.2 The particle morphology of nanosomes were determined using the Optical Microscopy (Olympus®, Japan). A drop of nanosome was added on a slide glass with a cover slip, and the nanosome was photographed through an optical microscope.

2.3.3.3 The entrapment efficiency of nanosomes was determined using dialysis technique (Trotta et al., 2002; Foco et al., 2005). For separating non-entrapped substance from nanosomes, 1 ml of nanosome dispersion were placed into a dialysis bag of cellulose acetate (dialysis membrane:cutoff at 3500 dalton, medium:distilled water, duration:16 hours), which was immersed in 300 ml water at room temperature and magnetically stirred at 30 rpm. The DPPH radical scavenging activity of nanosomes before and after dialysis were determined and the oil entrapment efficiency was calculated as follows:

$$\% \text{ entrapment} = \frac{innermembrane}{nanosome} * 100$$

Where innermembrane is the absorbance of entrapped materials in the nanosome formulations and nanosome denotes the absorbance of the initial concentration of materials.

2.3.4 Stability of rice bran oil nanosomes

Each of the rice bran oil nanosome formulations was evaluated for particle size and entrapment efficiency under heating and cooling cycle tests (1 cycle : -4°C, 24 hours and 45°C, 24 hours) for 5 cycles. The physical properties such as particle size, color and appearance were evaluated before and after the stability test. Furthermore, the stabilities of nanosome after storing at 4 ^{o}C for 2 months were also evaluated. The determination was performed in triplicate.

2.3.5 Antioxidant of rice bran oil nanosome after *in vitro* skin permeation test

2.3.5.1 Preparation of full-thickness newborn pig skin

The skin permeation studies were performed in modified Franz's diffusion cells (Hanson Research). Newborn pigs that had died of natural causes shortly after birth were obtained fresh from a local pig farm (Songkhla, Thailand). Although not a perfect model for human skin, the use of newborn pig skin is a practical alternative to the use of human skin for percutaneous absorption studies. In addition, pigs used in this study came from the local farm which is regulated by Department of Livestock Development, Thailand. Full thickness flank skins of newborn pigs weighing 1.4-1.8 kg were used. The

epidermal hair at the flank area was clipped with an electric hair clipper as close as possible to the skin without damaging it and excised with a scalpel. The subcutaneous fat and underlying tissues were carefully removed from the dermal surface. The skin was rinsed with phosphate buffer of pH 7.4, blotted dry, wrapped with aluminum foil and stored at -20°C for no longer than a month (Songkro *et al.*, 2003).

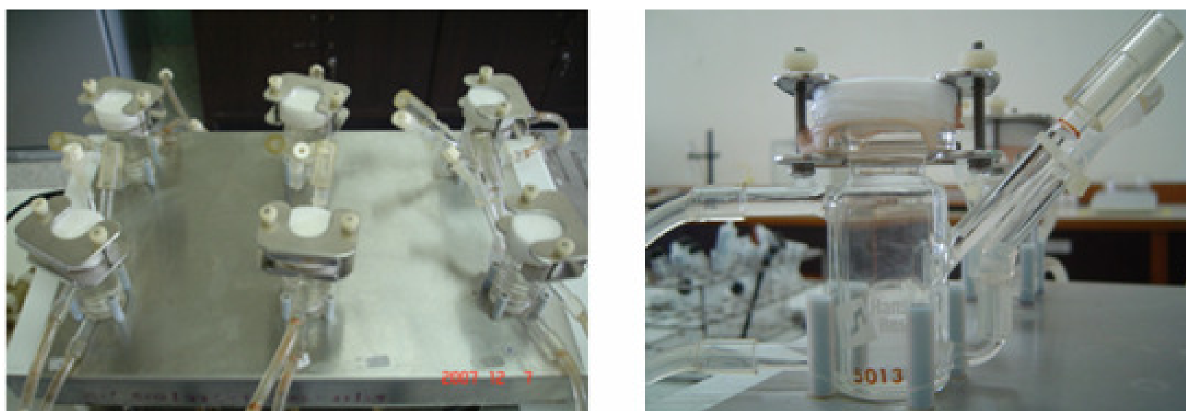

Figure 2.1 Modified Franz diffusion cell

2.3.5.2 *In vitro* skin permeation test procedure

The modified Franz diffusion cell was set at 37°C and magnetically stirred at 410 rpm. The skin samples were cut into 4.5 x 4.5 cm pieces, placed in phosphate buffer of pH 7.4 and hydrated at room temperature for 1 hour. The receptor compartment of the modified Franz diffusion cell was filled with 11 ml of phosphate buffer (0.1 M, pH 7.4). The diffusional surface area of the skin was 1.77 cm^2. Each of the rice bran oil nanosome formulations put in a donor compartment was placed on top of the skin.

Samples were withdrawn at 0.5, 1, 2, 4, 6, 8, 10 and 12 hours. An equal volume of fresh phosphate buffer was immediately added to the receptor solution after each sampling.

2.3.5.3 Antioxidant evaluation and data processing

Each samples (0.1 ml) was mixed into an equal volume of 6×10^{-5} M DPPH solution, 0.1 ml (in absolute ethanol) in 96-well plate. After stabilizing for 30 minutes at room temperature, the mixture was measured at an absorbance (A) of 520 nm by UV spectrophotometer. The measurement was done in triplicate. The prepared standard solution and control used in each experiment are as follows :

Control : 100 µl of phosphate buffer solution pH 7.4 + 100 µl of 6×10^{-5} M of DPPH in absolute ethanol

Control blank : 100 µl of phosphate buffer solution pH 7.4 + 100 µl of absolute ethanol

Sample : 100 µl of sample + 100 µl of 6×10^{-5} M of DPPH in absolute ethanol

Sample blank : 100 µl of sample + 100 µl of absolute ethanol

The % inhibition of each sample was calculated as previously described in section 2.3.2.1.

2.3.6 Acute dermal irritation test of nanosome

The test was performed following the test guideline (TG) No. 404: Acute dermal irritation/corrosion test of OECD guidelines for testing of chemical (2001). White rabbit (New Zealand) was used in this test. The epidermal hair on the back of rabbit was removed in the area of 10x10 cm^2. A 0.5 ml of sample was poured on the gauze patch size 2.5x2.5 cm^2 and applied on the hairless area of the back of rabbit by covering with the adhesive tape for 4 hours. 0.5 ml distilled water was used as control. A sample patch on the back skin area was protected from moving until reaching the time of observation. The sample patch was removed and the skin area of applying patch was cleaned. An irritation of skin was observed the level of erythema and oedema at time 1, 24, 48 and 72 hours or more than these when the irritation still occurred, but not over 14 days. The level of erythema and oedema were described in Table 2.2. This test was held by Thailand institute of scientific and technological research.

Table 2.2 Detail of level of erythema and oedema formation

Erythema and Eschar formation	level	Oedema formation	level
No erythema	0	No oedema	0
Very-slight erythema (barely perceptible)	1	Very-slight oedema (barely perceptible)	1
Well-defined erythema	2	Slight oedema (edges of area well-defined by definit raising)	2
Moderate to severe erythema	3	Moderate oedema (raised approximately 1 mm.)	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4	Severe oedema (raised more than 1 mm. and extending beyond the area of exposure)	4

2.3.7 Preliminary study for development of cream containing rice bran oil nanosome

2.3.7.1 Formulation of cream base

Oil in water emulsion cream bases were formulated with different materials and emulsifying agents, as shown in Table 2. All cream bases were prepared by the beaker method. Each material was accurately weighed and placed into two separate beakers, one for the oil phase and the other one for the water phase. Then, they were heated to about 70–75°C using a water bath. Once the desired temperature was reached, the water phase were slowly added to the oil phase with constant stirring. The mixture was stirred until congealed at room temperature. All cream bases were then evaluated for stability under a heating and cooling cycle tests (1 cycle : -4°C, 24 hours and 45°C, 24 hours) for 5 cycles and after storage at room temperature for 30 days. Their physical appearances including color, smoothness and phase separation were observed before and after the stability test. Cream bases with good appearances and promising stability were then selected for developing rice bran oil nanosome creams.

Table 2.3. Ingredients of the cream bases.

Ingredients	Content (g)			
	Rx 1	Rx 2	Rx 3	Rx4
Cetyl alcohol	-	2	-	4
Stearic acid	-	2	-	-
Cetostearyl alcohol	-	-	5	-
Hard paraffin	-	-	9	-
Glyceryl monostearate	-	3.8	-	-
Isopropyl myristate	-	2	4	-
Cremophor A6	-	1.8	-	-
Cremophor A25	-	1.8	-	-
Cetomacrogol 1000	-	-	4	-
Sepigel	10	-	-	-
Tween 80	-	-	-	1.39
Span 80	-	-	-	5.61
Lanol 1688	12	-	-	-
Mineral oil	5	0.8	-	8
Glycerin	10	10	6	-
Uniphen 0.5%	0.1	0.1	0.1	0.1
Purified water to	100	100	100	100

2.3.7.2 Formulation of creams containing rice bran oil nanosome

The creams of rice bran oil nanosome were prepared using suitable cream bases from section 2.3.7.1. Concentrations of nanosome were varied from 5 % to 20 % in order to evaluate for the formulation suitability. The nanosome creams were prepared in the same manner as described above. Rice bran oil nanosome was added into the cream bases at 50 ^{o}C with constant stirring. The mixture was stirred until congealed at room temperature.

2.3.7.3 Stability evaluation

Each formulations was evaluated for its physical stability under heating and cooling cycle tests (1 cycle: -4°C, 24 hours and 45°C, 24 hours) for 5 cycles. The physical appearances of cream formulations such as color, smoothness, phase separation as well as pH and viscosity were observed before and after the stability test. In addition, the antioxidant activity of the formulation was determined. The experiment was performed in triplicate.

2.3.7.3.1 pH determination

1 g of cream was diluted with 10 ml water. The pH of diluted cream was then determined using pH meter (Orion, Model 410A, USA). The measurement was performed in triplicate.

2.3.7.3.2 Viscosity assessment

The viscosity of each cream formulation was determined using Brookfield viscometer (Brookfieled dial reading, Model RVT, USA). The measurement was performed at the rotation speed of 5 rpm and using the spinder number 7. The measurement was performed in triplicate.

2.3.7.3.3 Antioxidant evaluation

One gram of each cream formulation was weighed and mixed with 10 ml of absolute ethanol. After sonicated for 30 minute, it was centrifuged for 10 minutes. The supernatant was then collected and adjusted to 25 ml with absolute ethanol. This solution was subsequently diluted to a concentration of 0.1 mg/ml and the antioxidant activity was determined using the DPPH radical scavenging assay as previously described in section 2.3.2.1.

2.3.7.4 The *in vitro* skin permeation studies

The modified Franz diffusion cell was set at 37°C and magnetically stirred at 410 rpm. The skin samples were cut into 4.5 x 4.5 cm pieces, placed in phosphate buffer of pH 7.4 and hydrated at room temperature for 1 hour. The receptor compartment of the modified Franz diffusion cell was filled with 11 milliliters of phosphate buffer (0.1 M, pH 7.4). The diffusional surface area of the skin was 1.77 cm^2. Two grams of each cream formulation was weighed in a donor compartment and placed on top of the skin. Sample was withdrawn at 0.5, 1, 2, 4, 6, 8, 10 and 12 hours each time. An equal volume of fresh phosphate buffer was immediately added to the receptor

solution after each sampling. After that, samples were determined for antioxidant activity using DPPH radical scavenging assay as previously described in section 2.3.2.1.

2.3.9 Statistical analysis

All experiment data were presented as mean ± SD. The relationship of the antioxidant activity and the DPPH assay was tested using linear regressing analysis and presented in term of its correlation coefficient (r^2). The stability test analysis was performed using the Student's t–test, with the significance level set at $p \leq 0.05$.

CHAPTER 3

RESULTS

3.1 Antioxidant activities of Rice bran oil

Free radical scavenging activity of antioxidant was studied using DPPH. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares and others, 1997). The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 520 nm, which is induced by antioxidant. Therefore, DPPH is used as a substrate to evaluate antioxidative activity of antioxidants (Duh and others, 1999).

Since oryzanol represents one of major compounds acting as primary antioxidant in rice bran oil, it was necessary to examine its contribution to the radical scavenging activity of the oil. For this, rice bran oils which contain low and high oryzanol were evaluated for their antioxidant activities. Due to the limit solubility of the oil, an organic solvent, ethanol:ether (1:2), was needed as its solvent in the DPPH assay. The results were illustrated in Table 3.1.

Table 3.1 : Antioxidant activities of Rice bran oil in ether : ethanol as solvent.

Type of rice bran oil	Concentration (w/v)	% inhibition n=3	EC_{50} (mg/ml) n=3
Low oryzanol	50	91.81 $\pm$ 0.40	7.12±1.495
	25	86.59 $\pm$ 1.40	
	5	37.88 $\pm$ 4.04	
	0.5	17.58 $\pm$ 2.65	
High oryzanol	50	93.50 $\pm$ 1.98	4.39±0.282
	25	64.04 $\pm$ 1.34	
	5	50.29 $\pm$ 1.38	
	0.5	14.95 $\pm$ 0.70	

The values are Mean ± S.D. (n = 3)

As shown in Table 3.1, the rice bran oils exhibited antioxidant effect with DPPH assay. The results from this assay indicated that rice bran oil achieves its antioxidant activity by acting as a free radical scavenger. The oil with high oryzanol (EC_{50} 4.39±0.282 mg/ml) showed higher antioxidant activity than the one with low oryzanol (EC_{50} 7.12±1.495 mg/ml). These results implied that oryzanol is likely to contribute to the radical scavenging activity of rice bran oil.

In addition, the comparative study on antioxidant activities of rice bran oil (with 0.04% oryzanol) and a commercial antioxidant, α-tocopherol or

vitamin E, was carried out. The dose response curves plotting between % DPPH radical scavenging and concentrations for rice bran oil and vitamin E are showed in Figure 3.1 and 3.2, respectively. The corresponding semi-logarithm plots were constructed in order to obtain the linear lines as shown in Figure 3.3 and 3.4, respectively. Consequently, their EC_{50} values were obtained by linear regression analysis of these linear plots. The results were summarized in Table 3.2.

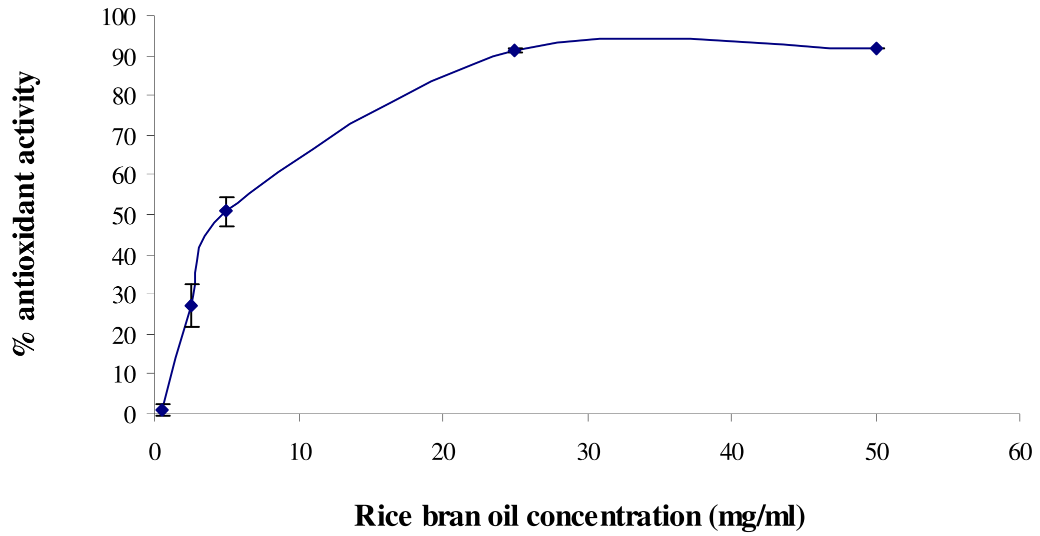

Figure 3.1 Correlation between rice bran oil concentration and its antioxidant activity determined by DPPH radical scavenging assay.

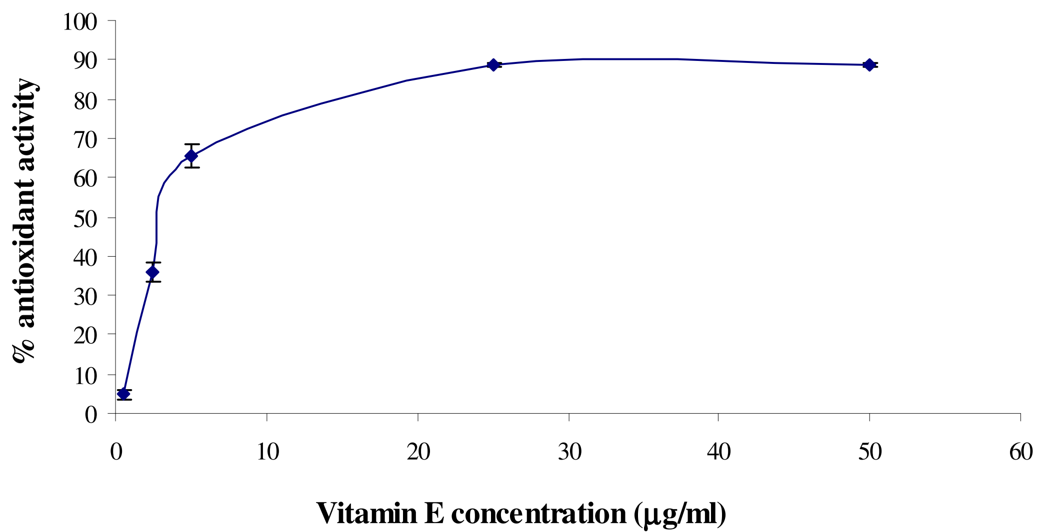

Figure 3.2 Correlation between vitamin E concentration and its antioxidant activity determined by DPPH radical scavenging assay.

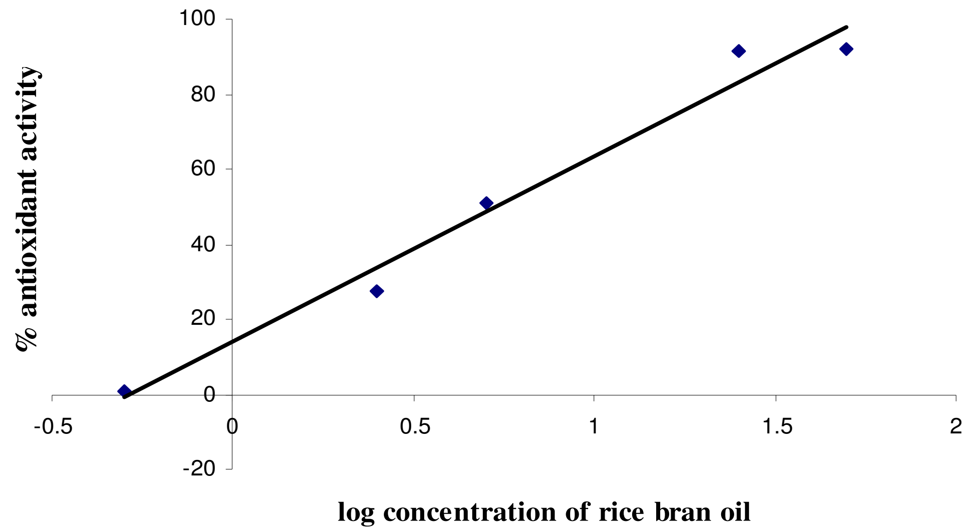

Figure 3.3 Correlation between log concentration of rice bran oil and its antioxidant activity determined by DPPH radical scavenging assay.

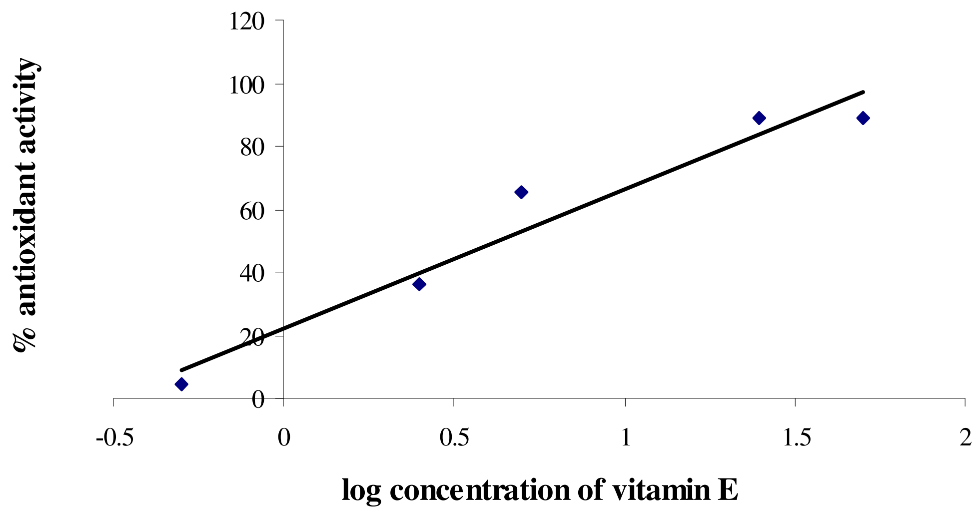

Figure 3.4 Correlation between log concentration of vitamin E and its antioxidant activity determined by DPPH radical scavenging assay.

Table 3.2 Comparision of antioxidant activities between Rice bran oil and vitamin E at various concentration.

Rice bran oil with oryzanol 0.04%			Vitamin E [a]		
Concentration (mg/ml)	**% antioxidant activity**	**EC_{50} (mg/ml)**	**Concentration (μg/ml)**	**% antioxidant activity**	**EC_{50} (μg/ml)**
50	91.74 ± 0.20	4.396 ± 0.282	50	88.68 ± 0.54	4.316 ± 0.196
25	91.28 ± 0.63		25	88.77 ± 0.37	
5	50.84 ± 3.56		5	65.49 ± 2.78	
2.5	27.27 ± 5.46		2.5	36.11 ± 2.45	
0.5	0.92 ± 1.59		0.5	4.76 ±1.28	

[a] Positive control

The values are Mean ± S.D. (n = 3)

Although, rice bran oil showed good activity in DPPH assay, it displayed remarkably low activity comparing to vitamin E. Its antioxidant activity was about 1000 fold less than that of vitamin E. Notice that the amount of oryzanol in the rice bran oil used in this study was only 0.04% or 4000 ppm. Therefore, the significant lower potency of this particular rice bran oil could be considered as a source of natural antioxidant, its antioxidant potency was not high enough to replace the synthetic one. The pharmaceutical technology and formulation development might, therefore, be necessary to improve its efficiency.

3.2 Preparation of rice bran oil nanosomes

Nanosomes of rice bran oil were prepared by reverse phase evaporation method. The composition of nanosomes were optimized in terms of type and ratio of both lipids and additives used. The results are shown in Table 3.3.

According to their physicochemical characteristics, three suitable formulations of rice bran oil nanosomes were obtains due to their good physical characteristics, the formulations include SPC:Tween 80 (84:16, weight ratio) with the total lipid of 30 and 50 μmol, SPC : CHOL : PEG 400 (7:2:1 molar ratio) with the total lipid of 30 μmol. All formulations showed good appearance of milky white colloidal solutions. The particle size of rice bran oil nanosome formulations were 269.6±21.06, 220.43±25.95 and 233.3±12.55 nm and the % entrapment efficiency were 54.21±8.12, 65.57±15.91 and 70.9±6.72,

respectively. These formulations will be used for further skin care product development.

Table 3.3 Physical characteristics of nanosome formulations.

Total lipid	Nanosome formulation	Physical appearance	Particle size (nm), n = 3	% Entrapment n = 3
20	EPC : CHOL : SA	Colloid pale yellow	603.3 ± 30.21	86.07 ± 5.78
	SPC : CHOL : PEG 400	Colloid milky white	270.3 ± 23.35	42.72 ± 6.88
	EPC : CHOL : PEG 400	Colloid pale yellow	409.6 ± 9.29	55.99 ± 6.06
	SPC : CHOL : PEG 4000	Colloid milky white	300.8 ± 8.55	49.67 ± 4.71
	EPC : CHOL : PEG 4000	Colloid pale yellow	470.63 ± 19.25	61.42 ± 13.26
	SPC : Tween 80	Colloid milky white	346.6 ± 1.23	48.6 ± 5.11
	EPC : Tween 80	Colloid pale yellow	248.7 ± 23.32	88.68 ± 46.57
30	SPC : CHOL : SA	Colloid milky white	609.12 ± 20.93	21.25 ± 6.74
	EPC : CHOL : SA	Colloid pale yellow	1398.67 ± 122.01	66.18 ± 10.98
	SPC : CHOL : PEG 400	Colloid milky white	233.3 ± 12.55	70.9 ± 6.72
	EPC : CHOL : PEG 400	Colloid pale yellow	566.07 ± 23.43	60.40 ± 11.86

Table 3.3 Physical characteristics of nanosome formulations (continued).

Total lipid	Nanosome formulation	Physical appearance	Particle size (nm), n = 3	% Entrapment n = 3
30	SPC : CHOL : PEG 4000	Colloid milky white	244.23 ± 6.83	42.69 ± 6.20
	EPC : CHOL : PEG 4000	Colloid pale yellow	1418.87 ± 36.78	58.11 ± 5.14
	SPC : Tween 80	Colloid milky white	269.6 ± 21.06	54.21 ± 8.12
	EPC : Tween 80	Colloid pale yellow	382.13 ± 15.01	57.54 ± 6.12
50	SPC : CHOL : SA	Colloid milky white	379.4 ± 16.37	83.87 ± 28.22
	EPC : CHOL : SA	Colloid pale yellow	288.23 ± 52.02	71.51 ± 12.47
	SPC : CHOL : PEG 400	Colloid milky white	438.33 ± 18.90	63.49 ± 14.96
	EPC : CHOL : PEG 400	Colloid pale yellow	374.57 ± 21.64	72.45 ± 10.59
	SPC : CHOL : PEG 4000	Colloid milky white	278.13 ± 24.21	51.6 ± 7.87
	EPC : CHOL : PEG 4000	Colloid pale yellow	651.7 ± 12.50	20.40 ± 8.16
	SPC : Tween 80	Colloid pale yellow	220.43 ± 25.95	65.57 ± 15.91
	EPC : Tween 80	Colloid pale yellow	355.13 ± 11.06	42.79 ± 5.63

The reproducibility of the three formulations were confirmed for their properties including physical appearance, particle size and % entrapment of nanosome formulation.

The results indicated that all formulations showed good appearance of milky white colloidal solution except the formulation with SPC:Tween (84:16) total lipid 50 μmol, of which a pale yellow color colloidal solution was observed as shown in Figure 3.5, 3.6 and 3.7.

Particle size of rice bran oil nanosomes ranging between 170-340 nm, were shown in Table 3.4, 3.5 and 3.6. The analysis of the particle size distribution is important for skin penetration since skin penetration is depended on their size. It becomes possible to forecast the potential of these vesicles penetrating the stratum corneum, in order to reach viable epidermis and dermis, where rice bran oil nanosome will develop its biological function.

Photomicrography of nanosome formulations showed that the nanosomes obtained in this work present spherical shapes with smooth, homogeneous surface and a non-aggregated distribution, as show in Figure 3.8. Their morphology is of fundamental importance because it can directly influence the penetration of the encapsulated active substances into the skin.

Table 3.4 Physical appearance, particle size and % entrapment of nanosome formulation; SPC:Tween 80 with the total lipid of 30 μmol

sample	Total lipid	Nanosome formulation	Physical appearance	particle size (nm)	% entrapment
1	30	SPC:TWEEN80	Colloid, milky white	217.87±64.19	73.20±4.51
2	30	SPC:TWEEN80	Colloid, milky white	179.73±8.37	68.35±5.99
3	30	SPC:TWEEN80	Colloid, milky white	210.63±90.01	71.63±1.65
Mean of 3 samples				202.74±20.25	71.06±2.47

Figure 3.5 Physical appearance of rice bran oil nanosome formulation; S:T30 = SPC:Tween80 with total lipid of 30 μmol, n=3

Table 3.5 Physical appearance, particle size and % entrapment of nanosome formulation; SPC:Tween 80 with the total lipid of 50 μmol

sample	Total lipid	Nanosome formulation	Physical appearance	particle size (nm)	% entrapment
1	50	SPC:TWEEN80	Colloid, pale yellow	341.6±43.41	51.70±2.76
2	50	SPC:TWEEN80	Colloid, pale yellow	339.93±32.44	55.31±10.80
3	50	SPC:TWEEN80	Colloid, pale yellow	291.93±24.38	59.14±11.97
Mean of 3 samples				324.49±28.21	55.38±3.72

Figure 3.6 Physical appearance of rice bran oil nanosome formulation. S:T50 = SPC:Tween80 (84:16) with total lipid of 50 μmol, n=3

Table 3.6 Physical appearance, particle size and % entrapment of nanosome formulation; SPC:CHOL:PEG 400 with the total lipid of 30 μmol

sample	Total lipid	Nanosome formulation	Physical appearance	particle size (nm)	% entrapment
1	30	SPC:CHOL:PEG 400	Colloid, milky white	206.17±42.62	87.03±8.78
2	30	SPC:CHOL:PEG 400	Colloid, milky white	197.23±31.10	81.71±3.76
3	30	SPC:CHOL:PEG 400	Colloid, milky white	239.4±28.99	75.36±6.02
Mean of 3 samples				214.27±22.22	81.36±5.84

Figure 3.7 Physical appearance of rice bran oil nanosome formulation. S:C:P30 = SPC:CHOL:PEG 400 (7:2:1) with total lipid of 30 μmol, n=3

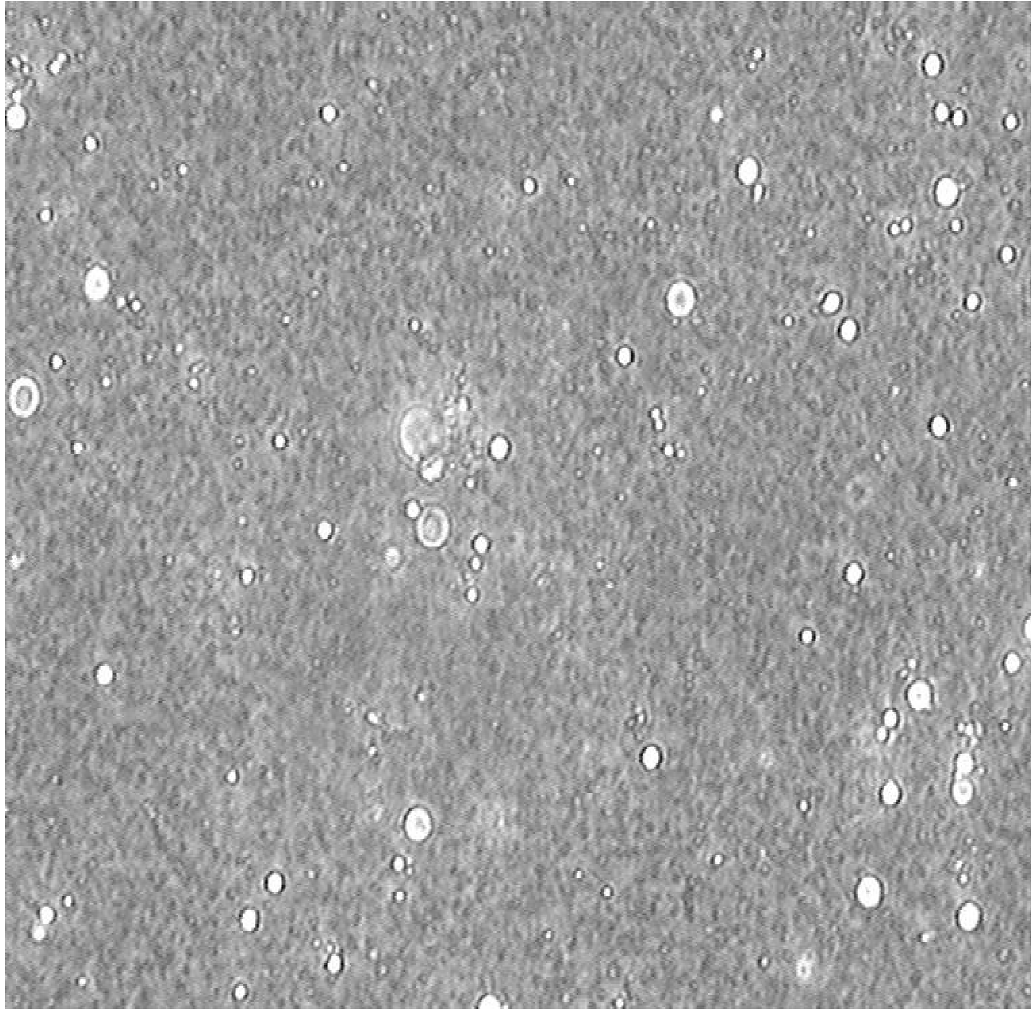

Figure 3.8 Optical microscopy image of nanosome formulation at 100x40 = 4000 magnification

3.3 Stability of rice bran oil nanosome

After freeze-thaw process, it was determined that the colour and appearance of all nanosome formulations were not different from the freshly prepared formulation. In addition, the particle sizes were also decreased except the formulation of SPC:Tween (84:16), total lipid 50 µmol where less viscosity and larger particle size was observed. The % entrapment of all nanosome formulations were decreased, as shown in Table 3.7. Furthermore, the stabilities of nanosome formulations after storing at 4 ^{o}C for 2 months were not significantly different. The entrapment efficiency of all nanosome formulations were significantly decreased ($p<0.05$), as shown in Table 3.7. The particle sizes of freshly prepared nanosome formulations as illustrasted in Table 3.7 were 202.74±20.25 to 324.49±28.21 nm. After storage for 2 months, their particle size were larger at a significant level ($p<0.05$).

Table 3.7 Physical appearance, particle size and % entrapment of rice bran oil nanosomes freshly prepare, after heating and cooling test and 4°C for 2 months.

Nanosome formulation	Freshly prepare			Freeze-thaw			4°C for 2 months		
	Physical appearance	**Particle size(nm)**	**% entrap n = 3**	**Physical appearance**	**Particle size(nm)**	**% entrap n = 3**	**Physical appearance**	**Particle size(nm)**	**% entrap n = 3**
SPC:Tween80 total lipid 30	Colloid, milky white	202.74± 20.25	71.06±2.47	Colloid milky white	169.03± 1.51	60.04±2.57	Colloid milky white	270.3± 23.35	66.71±19.37
SPC:Tween80 total lipid 50	Colloid, pale yellow	324.49± 28.21	55.38±3.72	Colloid pale yellow	385.76± 19.08	48.46±5.34	Colloid pale yellow	379.4± 16.37	49.67±4.71
SPC:CHOL: PEG 400 total lipid 30	Colloid, milky white	214.27± 22.22	81.36±5.84	Colloid milky white	109.4± 27.26	74.98±4.06	Colloid milky white	233.3± 12.55	75.37±4.36

The values are Mean ± S.D. (n = 3)

3.4 Antioxidant capacity of rice bran oil nanosome after skin permeation test (*in vitro* study)

The nanosome formulation with good passed the physical stability consistency in appearance was tested for animal skin permeation. A similar experiment was conducted using rice bran oil and the results were compared, by assessing the antioxidant activity. The results are shown in the Table 3.8.

Table 3.8 Antioxidant activity of rice bran oil permeating through the animal skin of each formulation at various time (hours).

Formulations	Antioxidant activity at various time (hours)							
	0.5	**1**	**2**	**4**	**6**	**8**	**12**	**Average**
Rice bran oil	6.92±1.23	3.89±1.11	5.64±3.67	10.60±0.67	19.00±0.84	16.23±3.58	16.13±1.84	11.20±5.96
SPC:Tween 80 Total lipid 30	29.96± 2.19	32.14±1.72	32.51±1.76	34.94±1.89	26.64±1.27	32.69±2.88	38.07±0.88	32.42±3.60
SPC:Tween 80 Total lipid 50	25.28±1.99	27.29±2.77	28.56±1.32	34.24±1.52	30.44±1.73	30.02±1.88	32.58±1.31	29.77±3.06
SPC:CHOL:PEG 400 Total lipid 30	44.47±2.17	46.20±3.30	53.42±2.64	54.15±0.35	49.22±1.89	50.05±1.56	51.14±1.09	49.81±3.55

The values are Mean ± S.D. (n = 3)

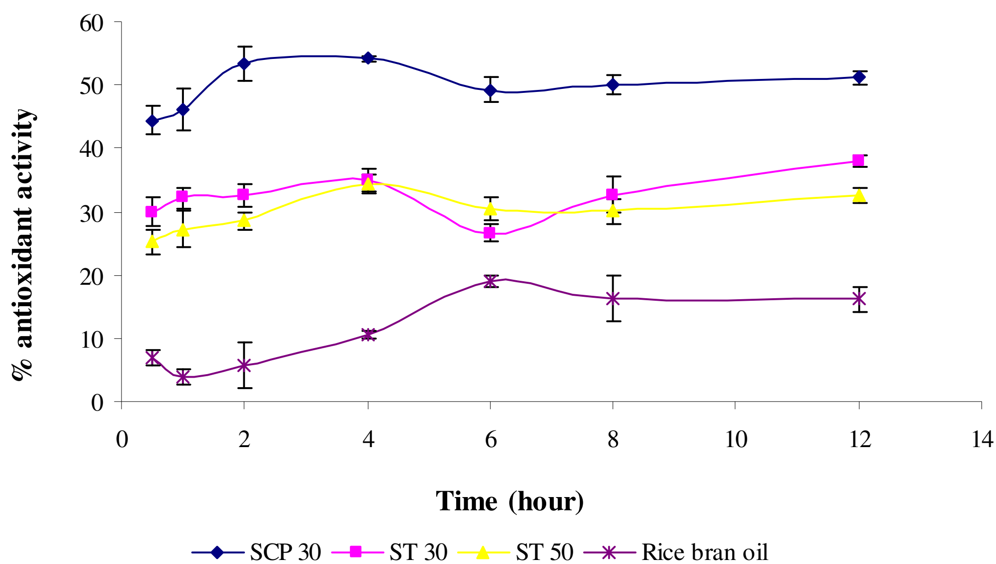

Figure 3.9 In vitro skin permeation of three formulations of rice bran oil and rice bran oil

As seen in Figure 3.9, all three nanosome formulations showed higher permeation abilities as compared to the rice bran oil at a significant level ($p<0.05$). The formulation of SPC:CHOL:PEG 400 (7:2:1) total lipid = 30 µmol showed the best skin permeability among all nanosome formulations at a significant level ($p<0.05$).

3.5 Acute dermal irritation test of nanosome

Nanosome formulation of SPC:Tween 80 with total lipid 30 µmol was chosen to be tested for acute dermal irritation because of its overall good appearance. The scores of dermal reaction were shown in Table 3.9.

Table 3.9 The scores of dermal reaction of the treated area on the skin of the rabbits treated with “rice bran oil nanosome”.

Rabbit No.	Scoring time (hours)							
	1		24		48		72	
	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
187	1	0	1	0	1	0	0	0
188	1	0	1	0	1	0	0	0
189	1	0	1	0	1	0	0	0

3.6 Preliminary study for development of cream containing rice bran oil nanosome

Four cream bases were prepared and tested for their stability by heating-cooling test method. The stability at room temperature for thirty days were also studied as described in section 2.3.7.1. The physical appearance, color, smoothness, phase separation, viscosity and pH were used as indexes stability for the selection of a suitable cream base (Table 3.10, Figure 3.10).

Table 3.10 General appearance, pH value and viscosity of the cream base

Formulation	Physical appearance	pH			viscosity ($\times 10^3$ cP)		
		before FT	After FT	After 30 days	Before FT	After FT	After 30 days
1	White viscous cream	5.14 ± 0.01	5.30 ± 0.02	5.22 ± 0.03	247.33 ± 0.29	302.33 ± 0.76	283.00 ± 0.00
2	White viscous cream	5.44 ± 0.06	5.37 ± 0.01	5.40 ±0.06	230.33 ± 0.76	201.67 ± 0.76	190.67 ± 1.04
3	White with low viscous cream	5.24 ± 0.02	5.30 ±0.05	5.27 ±0.03	115.67 ± 0.29	100.67 ± 0.29	100.67 ± 0.67
4	White with low viscous cream	5.32 ± 0.02	5.39 ±0.02	5.29 ±0.01	283.00 ± 0.00	369.00 ± 0.50	420.00 ± 0.00

The values are Mean ± S.D. (n = 3)

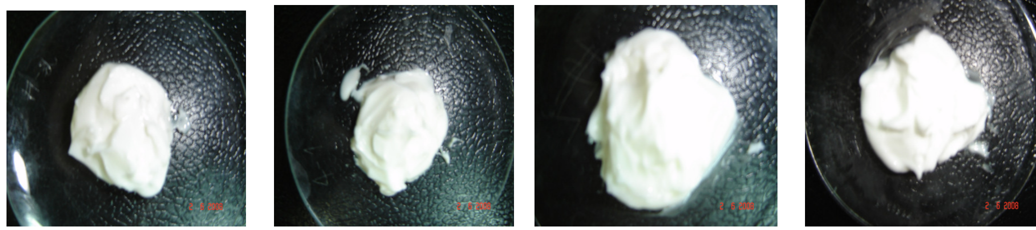

Figure 3.10 Cream base formulation 1-4 from left to right

Besides the viscosity and pH, the other physical appearances of cream bases were also assessed, such as smoothness, dispersion level. The results was shown in Table 3.11 along with these values obtained before and after freeze-thaw process.

Table 3.11 Assessment of the physical appearance of the cream base

Formulation	**Smoothness**			**Glossiness**			**Dispersion**		
	Before FT	After FT	After 30 day RT	Before FT	After FT	After 30 day RT	Before FT	After FT	After 30 day RT
Rx 1	4	4	4	4	4	4	4	4	4
Rx 2	4	3	3	4	4	4	3	3	3
Rx 3	3	2	3	3	3	3	4	4	4
Rx 4	3	3	3	4	4	4	3	3	3

The same assessment were also studied at room temperature for thirty days. It was found that these were no differences in physical appearance and consistency in all formulations after the test. The cream base with better appearance and pH 5.5-6.5, (cream base Rx 1) was closen to further formulate as rice bran oil nanosome cream.

3.7 Formulation of creams containing rice bran oil nanosome and stability evaluation.

Rice bran oil nanosome creams containing of 5%, 10% and 20% w/w nanosome were prepared from three suitable formulations of nanosome, SPC : Tween 80 (84:16, weight ratio) with the total lipid of 30 and 50 μmol, SPC : CHOL : PEG 400 (7:2:1 molar ratio) with the total lipid of 30 μmol.

Figure 3.11 Formulation of cream containing rice bran oil at various concentrations: 5%, 10% and 20%, respective concentrations (from left to right).

Figure 3.12 Formulation of cream containing rice bran oil nanosome (S:T 30) at various concentrations: 5%, 10% and 20%, respective (from left to right).

Figure 3.13 Formulation of cream containing rice bran oil nanosome (S:T 50) at various concentrations: 5%, 10% and 20% respective (from left to right).

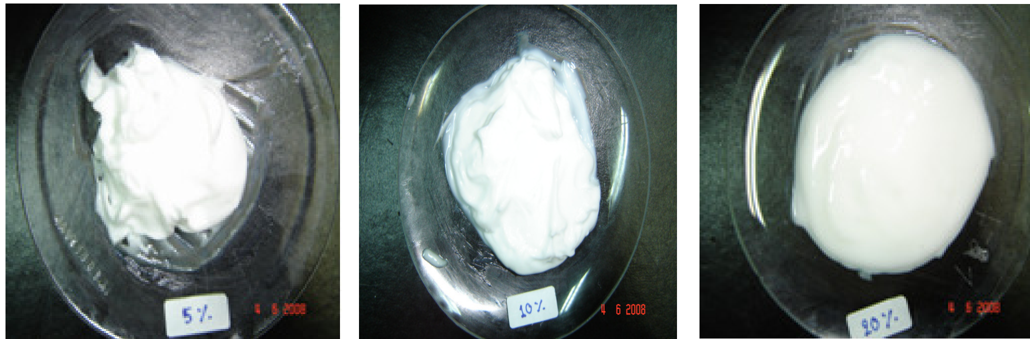

Figure 3.14 Formulation of cream containing rice bran oil nanosome (S:C:P 30) at various concentrations: 5%, 10% and 20% respective (from left to right).

Table 3.13 physical appearance, pH value and viscosity of each rice bran oil cream.

Formulations	Physical appearance	pH			viscosity ($\times10^3$ cP)		
		Before FT	After FT	After 30 days	Before FT	After FT	After 30 days
5% oil	White viscous cream	5.89 ± 0.006	5.72 ± 0.02	5.96 ± 0.06	202.67 ± 0.58	161.33 ± 0.29	257.33 ± 0.29
10% oil	White viscous cream	6.19 ± 0.03	5.97 ± 0.07	6.01 ± 0.08	209.33 ± 0.76	166.67 ± 0.29	269.33 ± 0.58
20% oil	White viscous cream	6.18 ± 0.03	6.24 ± 0.01	5.95 ± 0.01	198.67 ± 1.04	144.00 ± 0.50	214.67 ± 0.29
5% ST 30	White viscous cream	5.77 ± 0.01	6.91 ± 0.006	6.05 ± 0.02	162.67 ± 1.15	94.67 ± 0.76	173.33 ± 0.29
10% ST 30	White viscous cream	6.08 ± 0.006	6.71 ± 0.006	6.02 ± 0.01	124.00 ± 0.00	88.33 ± 0.29	70.67 ± 0.76
20% ST 30	White with low viscous cream	6.27 ± 0.05	6.86 ± 0.02	6.18 ± 0.02	40.00 ± 0.00	64.00 ± 0.00	32.00 ± 0.00
5% ST 50	White viscous cream	6.09 ± 0.01	5.82 ± 0.02	5.79 ± 0.01	165.33 ± 0.29	13.33 ± 0.29	56.00 ± 0.00
10% ST 50	White viscous cream	6.23 ± 0.02	6.80 ± 0.02	6.09 ± 0.02	120.00 ± 0.00	50.67 ± 0.29	64.00 ± 0.00
20% ST 50	White with low viscous cream	6.30 ± 0.01	5.37 ± 0.03	6.19 ± 0.02	37.33 ± 0.29	32.00 ± 0.00	32.00 ±0.00

Table 3.13 physical appearance, pH value and viscosity of each rice bran oil cream (continued).

Formulations	Physical appearance	pH			viscosity ($\times10^3$ cP)		
		Before FT	After FT	After 30 days	Before FT	After FT	After 30 days
5% SCP 30	White viscous cream	5.87 ± 0.03	6.10 ± 0.03	6.03 ± 0.02	173.33 ± 0.29	90.67 ± 0.29	135.20 ± 0.17
10% SCP 30	White viscous cream	5.78 ± 0.02	5.98 ± 0.04	6.08 ± 0.03	118.67 ± 0.29	36.00 ± 0.50	21.33 ± 0.29
20% SCP 30	White with low viscous cream	5.95 ± 0.04	6.05 ± 0.02	6.08 ± 0.02	32.00 ± 0.00	32.00 ± 0.00	32.00 ± 0.00

The values are Mean ± S.D. (n = 3)

All cream formulations were evaluated for physical appearance, consistency, viscosity and pH value before and after freeze-thaw process. The same assessment after storage at room temperature for thirty days were also studied as shown in Table 3.12. It has been found that all cream formulations have good physical appearance. After a heating and cooling test, it was found that the colors of all preparations were not changed. No phase separation was observed in all preparations. However, little changes of pH and viscosity were observed in all preparations. The pictures of rice bran oil nanosome creams and rice bran oil cream before and after heating and cooling test were shown in Figure 3.11, 3.12, 3.13 and 3.14, respectively.

The concentration of active substances increases, potency of antioxidant activity will also increase, as shown in Table 3.13. However, in comparision between rice bran oil nanosome cream and rice bran oil cream at equal concentration, it was found that antioxidant activity of rice bran oil cream was lower than rice bran oil nanosome cream. Moreover, the stability study at room temperature during one month period, rice bran oil cream with the concentration of 5% and 10% w/w of rice bran oil nanosome gave lower antioxidant activity than freshly prepare cream formulation.

Table 3.13 Antioxidant activity of various rice bran oil creams

Cream formulation	Antioxidant activity ± SD		
	Before Freeze Thaw	**After Freeze Thaw**	**After 30 days Room Temp.**
5% Oil	11.35 ± 0.63	10.55 ± 0.33	9.59 ± 0.35
10% Oil	16.28 ± 2.70	10.91 ± 0.62	10.52 ± 0.99
20% Oil	14.41 ± 2.89	12.71 ± 2.53	10.70 ± 0.66
5% ST 30	24.35 ± 0.79	23.12 ± 3.44	22.45 ± 2.45
10% ST 30	27.23 ± 1.21	20.18 ± 2.84	26.15 ± 0.93
20% ST 30	30.76 ± 1.06	32.31 ± 2.31	30.70 ± 1.57
5% ST 50	16.23 ± 3.58	15.97 ± 1.63	12.76 ± 0.56
10% ST 50	19.00 ± 0.84	19.99 ± 1.12	18.36 ± 0.89
20% ST50	22.70 ± 2.15	17.48 ± 1.17	19.64 ± 2.92
5% SCP 30	13.47 ± 0.79	13.97 ± 3.59	13.48 ± 1.38
10% SCP 30	36.90 ± 2.30	32.07 ± 2.43	35.26 ± 1.13
20% SCP 30	45.48 ± 4.52	47.02 ± 1.68	45.51 ± 3.94

3.8 Antioxidant capacity of rice bran oil nanosome cream after skin permeation test (*in vitro* study)

The rice bran oil nanosome creams with good physical properties and consistency in appearance were used on the *in vitro* skin permeation test. A similar experiment was performed by applying rice bran oil cream on pig skin and the results were determined, by assessing the antioxidant activity of rice bran oil cream permeating through the animal skin. The results are shown in the Table 3.14 and Figure 3.10. It was found that these were no significant differences of the antioxidant activities of the creams containing 5 and 10% nanosome compared to that of the commercial vitamin E cream.

Table 3.14 *In vitro* skin permeation studies of various rice bran oil cream with their % inhibition varied with time

Formualtion	Antioxidant activity ± SD at various time (hours) (%)							
	0.5	**1**	**2**	**4**	**6**	**8**	**12**	**Average**
5% oil	8.15±0.050	9.49±2.15	10.87±2.68	14.31±3.67	18.46±4.52	17.15±1.53	14.13±2.33	13.22±3.88
10% oil	5.07±1.56	7.49±2.16	12.18±0.57	13.47±2.04	17.45±1.67	22.35±0.55	19.01±1.60	13.86±6.22
20% oil	6.94±2.43	12.07±4.10	14.15±1.63	14.14±2.23	18.56±2.81	20.05±0.41	17.30±0.59	14.75±4.44
5% S:T 30	5.35±1.89	9.64±3.63	14.29±2.31	14.65±5.77	27.12±1.31	35.06±0.70	33.43±1.18	19.93±11.84
10% S:T 30	14.97±3.64	24.24±2.06	23.79±3.80	27.93±4.51	28.78±2.43	30.93±3.40	31.60±2.13	26.03±5.73
20% S:T 30	16.39±0.53	15.77±1.66	25.92±1.98	32.36±1.27	30.26±3.62	29.65±2.82	28.63±0.72	25.57±6.76
5% S:T 50	6.38±3.25	13.48±2.76	7.06±1.46	13.83±3.69	20.49±1.88	15.01±2.42	20.94±1.01	13.88±5.74
10% S:T 50	19.28±2.03	25.74±2.47	21.47±3.89	23.60±1.09	13.63±1.10	21.00±3.13	44.96±4.22	24.24±9.89
20% S:T 50	21.71±4.91	25.67±0.34	23.00±1.46	33.65±3.09	25.42±3.29	23.89±3.03	22.14±3.94	25.07±4.08

Table 3.14 *In vitro* skin permeation studies of various rice bran oil cream with their % inhibition varied with time (continued)

Formualtion	Antioxidant activity ± SD at various time (hours) (%)							
	0.5	**1**	**2**	**4**	**6**	**8**	**12**	**Average**
5% S:C:P 30	24.00±3.35	15.16±1.12	17.30±3.67	22.53±2.93	23.60±1.01	20.12±2.19	22.72±3.27	20.78±3.40
10% S:C:P 30	24.91±2.36	17.78±2.56	22.24±1.67	36.46±1.96	28.65±3.84	32.22±1.13	34.87±3.79	28.16±6.88
20% S:C:P 30	25.81±3.30	31.09±2.64	35.08±1.88	37.66±1.70	38.02±2.48	34.18±3.26	37.12±1.75	34.14±4.39
A commercial vitamin E cream	9.50±2.34	15.33±4.48	16.90±2.02	22.92±5.10	25.73±4.61	26.22±3.23	20.22±2.47	19.55±6.06

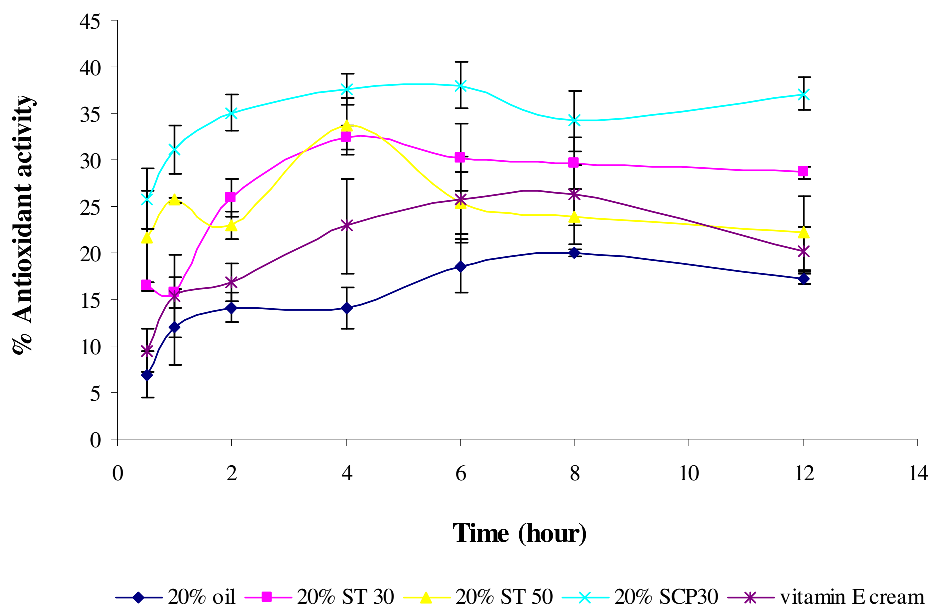

Figure 3.15 In vitro skin permeation of three formulation of rice bran oil nanosome cream, rice bran oil cream and commercial vitamin E cream

From Figure 3.15, it was shown that the antioxidant activity after skin permeation of the rice bran oil nanosome creams was higher than that of the rice bran oil cream at a significant level ($p<0.05$). Moreover, by comparing to the commercial vitamin E cream,it was found that 20% w/w creams containing rice bran oil nanosome show higher antioxidant activities, reflecting better skin permeation than the commercial vitamin E cream at a significant level ($p<0.05$). From these results, it is cleanly observed that the rice bran oil nanosome could provide better skin permeability. The rice bran oil nanosome cream containing nanosome formulation which composed of SPC:CHOL:PEG

400 (7:2:1) total lipid 30 μmol showed the best skin permeability at a significant level ($p<0.05$).

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Antioxidant activities of Rice bran oil

The results of the DPPH assay indicated that rice bran oil achieves its antioxidant activity by acting as a free radical scavenger. One of the major components that contributed to its radical scavenging activity is oryzanol. In the other words, the content of oryzanol seemed to be the most important factor that corresponded to the antioxidant activity of rice bran oil and its formulation. The source and manufacturing process of the oil should, therefore, be considered. The rice bran oil used in this study consisted of only 4000 ppm of oryzanol and the EC_{50} value of the oil was found to be 4.396 ± 0.282 mg/ml. By comparing to vitamin E, it was found that rice bran oil was less effective than vitamin E about 1000 times. This may due to the reason that rice bran oil used in this study was obtained from food industry and some substances including oryzanol might be removed during extraction process. Better source of rice bran oil should be taken into account for higher antioxidant capacity. The EC_{50} value of gamma-oryzanol has been reported in the study of Juliano *et al*. (2005) where the values was found to be 0.0603 mg/ml in the DPPH assay. However, it was still less antioxidant activity than alpha-tocopherol used as a reference antioxidant. This might be due to the fact that gamma-oryzanol appeared as a chain-breaking antioxidant which is less efficient than vitamin E which is able to scavenge organic radicals at lower

concentrations. However, comparing to BHT or BHA, gamma-oryzanol exhibited better action on free radical scavenging and had better protection effect against lipid peroxidation.

Though, the antioxidant property of rice bran oil is not exceptional, they could, however, enhanced by the association with other natural antioxidants. In Japan, rice bran oil has be popularly used as a skin and hair beauty enhancer for long time ago. It has been reported that rice bran oil is an effective substitute for lanolin, and can help reduce inflammation and soothe discomfort. It was also used in many purposes e.g., to cleanse, exfoliate, revitalize, prevent wrinkles, make the skin smooth and blemish free. (Natural's gift, 2008., Natural Japanese Beauty, 2008 and Oilsby Nature. Inc, 2008). Due to these applications, rice bran oil could be considered as a good candidate for natural antioxidant in cosmetic formulations. However, since the quantity of gamma-oryzanol plays an importance role on the activity of rice bran oil, its applications for cosmetic purpose need to be optimized in terms of the amount used and formulation design.

4.1.2 Preparation of rice bran oil nanosome and stability evaluation

Nanosomes containing rice bran oil can be formulated. The suitable formulations were obtained from SPC:Tween 80 (84:16 weight ratio), SPC:CHOL:PEG 400 (7:2:1 molar ratio) with the total lipid of 30 to 50 μmol. All formulations showed appropriate properties in both size and entrapment efficiency. However, the formulation of SPC seemed to be more stable than the formulation of EPC. This might be due to the difference in the composition of

lipid components between SPC (from soy bean) and EPC (from egg yolk). In fact, SPC is commonly more stable than EPC. However, after storage for two months, the nanosome vesicles could assimilate and coagulate to form larger vesicles. The formulations containing Tween 80 were easier to be formulated into nanosome than those containing cholesterol because nanosomes with cholesterol were more viscous. In addition, the sedimentation of lipid vesicles was observed if the ratios between phosphatidylcholine and cholesterol were not suitable. Moreover, the negative surface charge agent (e.g., PEG) could increase the entrapment efficiency of nanosomes. In all formulations, the entrapment efficiency of nanosomes was found to decreased about 10% from the initial value and its particle size was larger due to the coalescence effect after storage under 4°C for three months. The photomicrography of nanosome formulations showed lipid vesicles with spherical shapes and non-aggregation. Better skin permeation was observed in all nanosome formulations compared to the pure rice bran oil. This might be due to the small particle size of nanosome, as well as its bilayer structure that assist the deep penetration and distribution of rice bran oil (Manosroi *et al*., 2004). The intercellular space between corneocytes was reported to be about 0.1 μm (Higaki *et al*., 2003) in which nanosome with small size could access. On the other hand, nanosome with larger particle size than this gap can also be absorbed, since the elasticity of nanosome membrane allows the extrusion of nanosomes through this gap. Furthermore, incorporation of surfactant, Tween 80 or PEG 400 into the nanosome formulations could enhance skin permeation. The influence of nanosome components is not only or indeed the main factor operating skin

permeation but a definite additive effect with possible synergism in some cases, surfactants or ethanol with phosphatidylcholine (Gamal *et al*., 2000). Even though the formulation of SPC:CHOL:PEG 400 (7:2:1) with total lipid 30 μmol showed highest *in vitro* skin permeability, the formulation of SPC:Tween80 with total lipid of 30 μmol was chosen to be tested for acute dermal irritation because of its better properties in terms of overall physical appearances and entrapment efficiency. The results showed that within 1 hr after removal of the patches, all treated rabbits exhibited very slight erythema of skin (barely perceptible) and persisted until 48 hours of observation period. This might due to Tween 80, the surfactant in nanosome formulation, which can cause irritation to skin especially in case of deep penetration (Higaki *et al*., 2003). However, this skin reaction would have been recovered within 72 hours.

4.1.3 Formulation of creams containing rice bran oil nanosome

In the formulation development of rice bran oil nanosome cream, the oil in water cream base was selected based on the best physical appearances and characteristics in terms of color (white), smoothness, phase separation, viscosity and pH. In addition, it was physically stable under heating and cooling test and after storage at room temperature for thirty days. With this suitable cream base, the products of nanosome creams and rice bran oil creams came out in fine white colour cream with pH similar to human skin. The cream with 20% w/w of the nanosome showed less viscosity than the ones with 5% and 10% w/w. This relates to the higher amount of water in nanosome added into formulation resulting in lower viscosity of the product.

All cream formulations possessed good physical stability under freeze-thaw cycle test (6 cycles; 4°C and 45°C for a duration of 24 hours each). However, after storage at room temperature for one month, both 5% and 10% rice bran oil creams showed less antioxidant capacity.

The antioxidant capacity of rice bran oil nanosomes after *in vitro* skin permeation study was performed to evaluate the feasibility of the nanosome as a transport system of rice bran oil into the skin. The DPPH scavenging activity was used as an indirect index that reflects to the amount of entrapped rice bran oil delivered through the skin. The significant difference in the activity comparing between the nanosomes and the oil was observed only in the 20% formulation. At this concentration, all rice bran oil nanosome creams showed higher skin permeation than the oil at a significant level of $p<0.05$. It is also found that the 20% nanosome creams could permeate through the animal skin far better than a commercial vitamin E cream, at a significant level of $p<0.05$. Notice that the rice bran oil has less antioxidant activity that vitamin E. This implies that nanosome could deliver the entrapped substances into the skin with higher extent compared to the cream or emulsion based formulation (Egbaria and Weiner, 1990; Scheier and Bouwstra, 1994). In addition, it has been reported by several researchers that the high elasticity of vesicles could result in enhanced drug transport across the skin as compared to vesicles with rigid membranes (Planas *et al*., 1992; Sentjurc and Gabrijelcic, 1995; Cevc *et al*., 1998; Paul *et al*., 1998; van den Bergh *et al*., 1999; Guo *et al*., 2000a,b). It has been reported that nanosomes or liposomes with a heterogeneous lipid composition, or in other words, with several coexisting domains exhibiting different fluidity

characteristics in the bilayer (Vrhovnik *et al*., 1998) leading to the enhancement of penetration of entrapped substances into the skin. It has also been supposed that once in contact with skin, some budding of liposomal membrane might occur (Cevc *et al*., 1995; Vrhovnik *et al*., 1998). This could cause a mixing of the nanosomes or liposome bilayer with intracellular lipids in the stratum corneum (Egbaria *et al*., 1991), which may change the hydration conditions and thereby the structure of lipid lamellae. This may enhance the permeation of the lipophilic drug into the stratum corneum and ease the diffusion of hydrophilic drugs into the interlamellar spaces. In our study, the nanosome cream formulation SPC:CHOL:PEG 400 (7:2:1); total lipid = 30 μmol showed the best skin permeability at a significant level ($p<0.05$).

4.2 Conclusion

From the result , it could be conclude that rice bran oil is a free radical scavenger. Although it is less effective than commercial antioxidants such as vitamin E, rice bran oil can be considerate as one of the good sources for natural antioxidants. In addition, rice bran oil possesses several benefits for cosmetic purposes such as cleansing, revitalizing as well as wrinkle prevention. Currently, rice bran oil is still the center of interest for cosmetic development.

The nanosomes of rice bran oil for improving the skin permeability were developed in this study. The suitable formulations were found as SPC:Tween 80 (84:16 weight ratio), SPC:CHOL:PEG 400 (7:2:1 molar ratio) and the total lipid were in range of 30 to 50 μmol. All formulations showed appropriate properties in both size and entrapment efficiency. The *in*

vitro skin permeation were significantly higher than that of pure rice bran oil. The highest skin permeation was observed in nanosome with SPC:CHOL:PEG 400 (7:2:1) total lipid 30 μmol.

In the formulation development, rice bran oil nanosome cream contaning 5%, 10% and 20% w/w of each nanosome and rice bran oil cream with the same concentration were prepared. All nanosome creams showed good stability and higher antioxidant activity effect comparing to the rice bran oil creams. In addition, it was obvious that the *in vitro* skin permeation of all nanosome formulations were significantly higher than that of rice bran oil cream. The best results on skin permeability was observed on the nanosome cream containing SPC:CHOL:PEG 400 (7:2:1) with total lipid 30 μmol. In addition, it was revealed that our nanosome could favorably deliver the entrapped antioxidant substances of rice bran oil into the skin with higher efficiency than the commercial vitamin E product.

Overall, rice bran oil could be considered as a good natural antioxidant. Since its antioxidant properties are not exceptional as comparing to the commercial antioxidant like vitamin E, the enhancing of its activity should be considered by either improving the quality of oil or mixing with other natural antioxidants. The applying pharmaceutical technology such as nanosome could obviously improve the potential of using rice bran oil as cosmetic ingredients in terms of the feasibility of both product development and skin penetration. The product of rice bran oil nanosome are therefore, promising to used for both cosmetic and cosmeceutical products.

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VITAE

Name Miss Pariporn Maharachatahtakul

Student ID 4910720039

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Engineering	Prince of Songkla University	2005

List of Publication and Proceeding

Poster presentation, Ingkatawornwong, S., Pinsuwan, S., Worachotekanijorn, K., Maharachatahtakul, P., Amnuaikit, C. (2007). Development of rice bran oil liposome for using in cosmetics. The 48th Annual Meeting of Society for Economic Botany: Symposium: Plants in the Service of Human Health: Continuing the search for new plant-based therapies, Lake Forest College-Marriot Courtyard, Lake Cook Rd. 4-7 June 2007. Chicago, Illinois, USA.

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