CHAPTER 3

RESULTS AND DISSCUSSION

The results of this study were divided into 6 parts as the followings:

Part 1: Crude extracts preparation and their biological activities

Part 2: Semi-purified fractions preparation from the crude extracts and their *in vitro* biological and anti-hair loss activities

Part 3: Development of the blank neutral niosomes and the niosomes loaded with *O. sativa* crude extract by chloroform film method and $scCO_2$

Part 4: Development of blank cationic niosomes and cationic niosomes loaded with the rice (*Oryza sativa*) bran semi-purified fraction (OSF3) containing the unsaturated fatty acids by $scCO_2$ technique

Part 5: *In vitro* transfollicular penetration of unsaturated fatty acids of gel OSF3 niosomes in porcine skin by Franz diffusion cells

Part 6: *In vivo* hair growth promotion activity of gel containing cationic niosomes loaded with OSF3

Part 1: Crude extracts preparation and their biological activities

1.1 Optimization of the extraction condition

The bran of *O. sativa* (rice bran) was selected to be the representation for the optimization of the extraction conditions, $scCO_2$ and maceration method.

1.1.1 Percentage yields of the extracts

For the preparation of the rice bran extract by $scCO_2$, the pressure at 200 bar was used because it has been reported that the solubilities of oleic acid which were the main unsaturated fatty acid at 200 bar/40 °C and 300 bar/40 °C were 4.1 and 3.64 times of triolein (Brunetti et al., 1989). The extraction temperature was set at 40°C because of the concern of chemical stability of the unsaturated fatty acids. When the temperature increased from 40 to 60°C, the contents of the fatty acids decreased by 4.8%, owing to the degradation of the fatty acids (Shen et al., 1996). The percentages of ethanol (co-solvent) in the $scCO_2$ system were varied from 10-35 % w/v in order to determine the suitable co-solvent concentrations **(Table 9)**. The median concentration of ethanol as a co-solvent (25% w/v) from this range (10-35 % w/v) was selected for further study since there was no significant difference of the unsaturated fatty acid contents and the percentage yields in this ethanol concentration range. The extraction was performed for 4 times since several studies have revealed that free fatty acid contents were concentrated in the first of the four fractions from the repeat 4 extraction times by $scCO_2$ (Shen et al., 1996; Zhao et al., 1987). Therefore, the conditions for the $scCO_2$ extraction of rice bran were 200 bar, 40°C and 25% w/v of 95% v/v ethanol.

Table 10 indicated the percentage yields of the extracts prepared by the $scCO_2$ technique and ethanolic maceration. The yields from the ethanolic maceration extraction (17.90 ± 0.67 % w/w) were significant higher than those from the $scCO_2$ with 25% w/v of 95% v/v ethanol as a co-solvent (11.21 ± 0.54 % w/w) ($p<0.05$). This may be due to the more exposure time of the rice bran raw materials to the

solvent in the maceration method (24 hours) than the $scCO_2$ (8 hours). As known, the extraction yield and compositions depend on the extracting conditions especially

Table 9 Comparison of the percentage yields, unsaturated fatty acid contents of the rice bran extracts prepared by $scCO_2$ process with various concentrations of 95% v/v ethanol as a co-solvent

concentrations of 95% ethanol (%w/v)	percentage yields (%w/w)	fatty acid contents in the extract (%w/w)		
		gamma-linolenic acid	linoleic acid	oleic acid
50	11.84	4.94	23.33	26.20
33	10.48	5.66	22.11	25.11
25	11.21	5.67	23.62	27.28
20	11.37	5.40	24.01	28.15
10	12.60	5.52	22.50	27.29

the extraction time (Sahena et al., 2009). Moreover, the temperature at 40 ± 1^{o}C was used in the $scCO_2$ method while 27 ± 2^{o}C (room temperature) was used in the ethanolic maceration that some heat labile components may be destroyed by high temperature exposure in the $scCO_2$ process. Also, rice bran may contain more polar compounds than non-polar compounds, which can be extracted more from the maceration method (the more polar solvent of 100% w/v of 95% v/v ethanol) than the $scCO_2$ method (the less polar solvent of 25% w/v of 95% v/v ethanol as a co-solvent).

Table 10 Comparison of the percentage yields, unsaturated fatty acid contents and total phenolic contents of the rice bran extracts prepared by the two non- heated processes ($scCO_2$ with 25% w/v of ethanol and maceration in 95% v/v ethanol)

sample	percentage yields (%w/w)	fatty acid contents in the extract (%w/w)			total phenolic contents (gallic acid equivalent; GAE mg/g of the extract)
		gamma-linolenic acid	linoleic acid	oleic acid	
rice bran extract from $scCO_2$ with 25% w/v ethanol	11.21± 0.54	5.67± 0.52	23.62± 2.62	27.28± 1.22	0.65± 0.05
rice bran extract from maceration	17.90± 0.67	4.41± 0.73	20.03± 1.89	19.48± 1.78	0.51± 0.07

Values represent mean ± S.D. (n=3)

1.1.2 Unsaturated fatty acid and the total phenolic contents (TPC) in the rice bran extracts

The rice bran extract from the $scCO_2$ technique gave gamma-linolenic acid, linoleic acid and oleic acid contents at 5.67 ± 0.52, 23.62 ± 2.62 and 27.28 ± 1.22 % w/w, respectively, which were more than those from the maceration method which gave 4.41 ± 0.73, 20.03 ± 1.89 and 19.48 ± 1.78% w/w, respectively **(Table 10)**. The contents of gamma-linolenic acid (C18:3) from these two methods were higher, while the those of linoleic acid (C18:2) and oleic acid (C18:1) were lower than the concentration ranges of these unsaturated fatty acids found in raw rice bran reported previously (Chakrabarty, 1989; Sayre and Saunders, 1990). This may be due to the different sources of the raw rice bran, the extraction methods and the periods of the harvesting. TPC in the form of gallic acid in the extracts from the $scCO_2$ method and ethanolic maceration were 0.65 ± 0.05 and 0.51 ± 0.07 mg/g, respectively **(Table 10)**. Although the maceration extraction which was 3 times (24/8) longer exposure time of the raw rice bran to the solvent gave higher percentage yields than the scCO2 technique, it gave lower content of unsaturated fatty acids than the $scCO_2$ technique. The higher unsaturated fatty acids and TPC in the $scCO_2$ extract might be due to the higher lipophilic property of $scCO_2$ than ethanol. Although 100% w/v of 95% v/v ethanol in the maceration was more polar than the 25% w/v of 95% v/v ethanol in the $scCO_2$ method, the high solvating power of $scCO_2$ gave more influence on the extraction of unsaturated fatty acids (Sahena et al., 2009). Thus, the C18:3 acids which were higher polarity than C18:2 and C18:1, respectively, can be dissolved more by the high solvating power of $scCO_2$. Moreover, $scCO_2$ can dissolve more fatty acids and phenolic compounds especially at the pressure of 80-250 bar and at the

temperature range of 40-80°C (Chrastil, 1982). It has also been reported that $scCO_2$ can extract higher contents of lipids and fatty acids than by chloroform, methanol and ethanol Soxhlet extraction (Cheung et al., 1998).

1.1.3 Antioxidative activity of the extracts

1.1.3.1 DPPH radical scavenging activity

Antioxidative activities of the rice bran extracts determined by DPPH radical scavenging were shown in **Table 11**. The SC_{50} of the extracts by $scCO_2$ (SC_{50} = 6553.11 ± 561.31 μg/ml) and ethanolic maceration (SC_{50} = 6792.12 ± 605.51 μg/ml) exhibited no significant different scavenging activities ($p<0.05$). Although the extracts by these two extraction methods gave lower activities than the standard vitamin C (SC_{50} = 44.67 ± 4.23 μg/ml) and vitamin E (SC_{50} = 39.56 ± 2.31 μg/ml) of about 150 times, they exhibited higher activities than the standard unsaturated fatty acids, gamma-linolenic acid (SC_{50} = 15491.23 ± 22.31 μg/ml), linoleic acid (SC50 = 39825.45 ± 51.23 μg/ml) and oleic acid (SC_{50} = 55584.67 ± 918.23 μg/ml) of about 2, 6 and 9 times, respectively. Therefore, although the extract by $scCO_2$ gave higher contents of unsaturated fatty acids and TPC than the maceration method, these two extraction methods appeared not to affect the antioxidant activities of the extracts. In fact, many bioactive scavenger compounds including phenolic acids, ferulic acid, vanillic acid, protocatechuic acid, phenolic acid ester, gamma-oryzanol, phytic acid and inositol have been found in rice bran (Lee et al., 2008). Hence, the DPPH radical scavenging activity may be not only from the unsaturated fatty acids and gallic acid, but also from other phenolic bioactive compounds. These phenoilc compounds may interrupt with the free-radical chain of oxidation and donate

Table 11 Comparison of biological activities (antioxidative, tyrosinase inhibition and the stimulation index (SI) on the 30th passage aged human skin fibroblasts) of the rice bran extracts prepared by the scCO2 (with 25% w/v of 95% v/v ethanol as a co-solvent) and maceration in 95% v/v ethanol

sample	SC_{50}[a] (ug/ml)	IPC_{50}[b] (ug/ml)	CC_{50}[c] (ug/ml)	IC_{50}[d] (ug/ml)	Stimulation index (at 0.1 mg/ml)
rice bran extract from scCO2	6553.11± 561.31	1248.54± 51.23	68229.56± 8062.25	25982.15± 1342.25	1.10 ± 0.12
rice bran extract from maceration	6792.12± 605.51	1444.18± 182.11	75043.24± 5134.24	48065.14± 4141.56	1.14 ± 0.15
vitamin C	44.67± 4.23	74.21± 40.23	-	-	1.20 ± 0.08
vitamin E	39.56± 2.31	34.56± 16.24	-	-	-
EDTA	-	-	152.23± 39.78	-	-
kojic acid	-	-	-	24.23± 2.56	-
gamma-linolenic acid	15491.23± 22.31	1931.52± 36.25	1745.12± 32.85	775.23± 15.85	-
linoleic acid	39825.45± 51.23	1254.14± 11.56	14791.45± 113.74	2554.23± 81.85	-
oleic acid	55584.67± 918.23	3513.16± 41.87	26345.36± 234.23	7113.45± 45.56	-

Values represent mean ± S.D. (n=3)

[a] SC_{50}=scavenging concentration at 50% activity (ug/ml)

[b] IPC_{50}=inhibition peroxidation concentration at 50% activity (ug/ml)

[c] CC_{50}=chelating concentration at 50% activity (ug/ml)

[d] IC_{50}=tyrosinase inhibition concentration at 50% activity (ug/ml)

hydrogen from phenolic hydroxy groups, thereby forming stable free radicals, which do not initiate or propagate further oxidation reaction (Sherwin, 1978).

1.1.3.2 Lipid peroxidation inhibition activity

The rice bran extracts prepared by $scCO_2$ and ethanolic maceration did not show any significant differences of lipid peroxidation inhibition at the IPC_{50} values of 1248.54 ± 51.23 and 1444.18 ± 182.11 μg/ml, respectively (p<0.05) (**Table 11**). Although all extracts showed lower lipid peroxidation inhibition than the standard vitamin C (IPC_{50} = 74.21 ± 40.23 μg/ml) and E (IPC_{50} = 34.56 ± 16.24 μg/ml) of about 19 and 43 times, respectively, they exhibited similar activity to the standard linoleic acid (IPC_{50} = 1254.14 ± 11.56 μg/ml), but higher activity than gamma-linolenic acid (IPC_{50} = 1931.52 ± 36.25 μg/ml) and oleic acid (IPC_{50} = 3513.16 ± 41.87 μg/ml) of about 2 and 3 times, respectively. The lipid peroxidation inhibition of the extracts might be from the unsaturated fatty acids and the TPC which can inhibit the peroxidation formation. This result has been confirmed by the previous study that the rice bran extract has a strong antioxidant capacity and also lipid peroxidation inhibition activity (Parrado et al., 2003).

1.1.3.3 Metal ion chelating assay

The rice bran extracts prepared by $scCO_2$ and ethanolic maceration gave no significant difference of metal ion chelating activities with the CC_{50} values of 68229.56 ± 8062.25 and 75043.24 ± 5134.24 μg/ml, respectively (p<0.05) (**Table 11**). The CC_{50} values of the extracts were lower than the standard EDTA (CC_{50} = 152.23 ± 39.78 μg/ml) and the standard unsaturated fatty acids, gamma-linolenic acid (CC_{50} = 1745.12 ± 32.85 μg/ml), linoleic acid (CC_{50} = 14791.45 ± 113.74 μg/ml) and oleic acid (CC_{50} = 26345.36 ± 234.23 μg/ml) of about

466, 40, 5 and 3 times, respectively. The poor chelating activities of the extracts might be from the low contents of the chelating agents, such as tannin and citric acid, thereby providing weak competitive inhibition to form a complex with the ferrous instead of the ferrozine (Perez et al., 2009).

1.1.4 Tyrosinase inhibition activity

The rice bran extract by the $scCO_2$ showed tyrosinase inhibition activity at IC_{50} value of 25982.15 ± 1342.25 μg/ml which was about 2 times higher than that by the $scCO_2$ process (IC_{50} = 48065.14 ± 4141.56 μg/ml), but lower than the standard kojic acid (IC_{50} = 24.23 ± 2.56 μg/ml) of about 1000 times (**Table 11**). The standard unsaturated fatty acids, gamma-linolenic acid (IC_{50} = 775.23 ± 15.85 μg/ml), linoleic acid (IC_{50} = 2554.23 ± 81.85 μg/ml) and oleic acid (IC_{50} = 7113.45 ± 45.56 μg/ml) exhibited higher activity than the extracts of about 40, 14 and 5 times, respectively. The low tyrosinase inhibition of the extracts in comparing to the standards might be from not only the low contents of the compounds which have specific configuration to inhibit the tyrosinase enzyme and regulate the redox of l-DOPA and dopaquinone, but also the low contents of chelating agents that can bind to the copper ion in the active sites of the enzyme (Maisuthisakul and Gordon, 2009). This result agreed with the low metal ion chelating activity of the extract.

1.1.5 Cell proliferation activity on aged human skin fibroblasts of the extracts

The rice bran extracts prepared by both extraction processes were investigated for human skin fibroblast proliferation by SRB assay. The stimulation index (SI) values of the extracts at 0.1 mg/ml on aged normal human skin fibroblasts (30^{th} passage) were shown in **Table 11**. The concentrations of the extracts at 0.1

mg/ml were used because this concentration was the maximum solubility of the extract in the aqueous cell growth medium. The SI values of the $scCO_2$ and the ethanolic maceration extracts and the standard vitamin C were not significant different (p<0.05) at 1.10 ± 0.12, 1.14 ± 0.15 and 1.20 ± 0.08, respectively. This has indicated not only the similar human skin fibroblast stimulation effect of the extracts prepared by the two extraction processes to vitamin C, but also no cytotoxicity of the extracts on the aged normal human fibroblast was observed as well. This result has also anticipated the stimulation effects of these two extracts on collagen synthesis of the aged human skin fibroblasts, which may be beneficial for anti-hair loss property.

1.2 Extraction of the selected 10 plants

1.2.1 Unsaturated fatty acid and total phenolic contents in the crude extracts

The crude extract from *C. tinctorius* by both ethanolic maceration and $scCO_2$ showed the highest percentage yields at 33.88 ± 3.67 and 20.23 ± 1.65 % (w/w), respectively **(Table 12)**. Although, the ethanolic maceration of most plants gave higher percentage yields than the $scCO_2$ technique, it gave lower contents of the main target compounds (unsaturated fatty acids) than that from the $scCO_2$ technique. The maceration technique gave more impurities than that from the $scCO_2$ technique. In fact, the polar organic solvents such as methanol and ethanol can also extract many polar compounds such as phenolic compounds, flavonoids and anthraquinone (Lou et al., 1996), thereby giving the extract with higher impurities and percentage yields. The high lipophilic property and solvating power of the $scCO_2$ fluid can dissolve the unsaturated fatty acids especially at the pressure of 80-250 bar and the temperature range of 40-80°C (Chrastil, 1982). Thus, the $scCO_2$ fluid appears to be the best solvent choice to obtain the extract with high content of unsaturated fatty acids.

Table 12 Comparison of the percentage yields, unsaturated fatty acid contents and the total phenolic contents of the ten edible plant crude extracts prepared by the two non- heated processes ($scCO_2$ and ethanolic maceration)

Plants		**percentage yields (%w/w)**		**unsaturated fatty acid contents (%w/w)**						**total phenolic contents (gallic acid equivalent; GAE mg/g of the extract)**	
scientific name	**common name**			gamma-linolenic acid		linoleic acid		oleic acid			
		$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration
Arachis hypogaea L.	Peanut	16.94	12.88	-	-	0.17	1.08	0.24	0.72	0.33	0.39
Carthamus tinctorius L.	Safflower	20.23	33.88	9.36	4.45	4.02	2.13	0.65	0.03	1.36	1.44
Glycine max (L.) Merr.	Soybean	5.42	26.28	0.14	0.05	1.08	0.56	0.12	0.08	0.53	0.46
Helianthus annuus L.	Sunflower	14.01	29.93	0.02	0.01	1.97	0.64	0.42	0.82	2.41	1.76
Linum usitatissimum L.	Flax	7.17	12.14	0.28	0.31	0.63	0.77	0.19	0.11	0.43	0.70
Nelumbo nucifera Gaertn.	Lotus	3.34	2.47	0.08	0.11	1.07	2.45	0.19	0.62	0.42	0.58
Oryza sativa L.	Rice	11.21	17.90	5.67	4.41	23.34	20.03	27.28	19.48	0.65	0.51
Sesamum indicum L.	Sesame	12.01	15.88	0.05	0.09	3.96	3.19	2.75	1.89	0.71	0.86
Sorghum bicolor (L.) Moench	Sorghum	2.54	5.12	0.02	0.15	5.56	2.66	1.78	0.51	1.05	1.13
Zea may L.	Corn	4.55	14.97	0.04	0.05	0.57	1.09	0.2	0.52	0.76	0.96

Note: Values represented mean (n=3)

"-"represented not found in the sample

Total phenolic contents were presented as gallic acid equivalent (GAE) mg/g of the crude extracts.

For other methods such as Soxhlet extraction and maceration, they require large amount and high purity of organic solvents (chloroform , hexane or methanol) that are hazardous and can be the flammable solvent wastes and are generally cumbersome (Sahena et al., 2009). Moreover, the Soxhlet extraction was the non-selective, time consuming and toxic fume emission technique which is harmful to the environments (Naudé et al., 1998). The crude extract of *O. sativa* by both processes exhibited the highest contents of all unsaturated fatty acids, except gamma-linolenic acid in comparing to the crude extracts of *C. tinctorius.* The *O. sativa* crude extract from the $scCO_2$ technique contained the gamma-linolenic acid, linoleic acid and oleic acid contents at 5.67 ± 0.52, 23.62 ± 2.62 and 27.28 ± 1.22% (w/w), which were more than those from the maceration method (4.41 ± 0.73, 20.03 ± 1.89 and 19.48 ± 1.78%, w/w) of 1.29, 1.18 and 1.40 times, respectively. Moreover, by $scCO_2$ the *O. sativa* crude extract exhibited the highest total unsaturated fatty acids (TUC) at 56.57 ± 3.73% (w/w) which was higher than those in the *C. tinctorius* (14.03 ± 2.17%, w/w) and *S. bicolor* (7.36 ± 1.18%, w/w) crude extract of 4.5 and 8.5 times, respectively. The crude extracts which gave the highest total phenolic contents (TPC) in the form of gallic acid were observed in *H. annuus* from both by the $scCO_2$ and the maceration methods at 2.41 ± 0.13 and 1.76 ± 0.07 mg gallic acid equivalent /gram of the crude extract (GAE mg/g), respectively. In fact, various phenolic compounds were found in *H. annuus*, such as 5-*O*-caffeoylquinic acid, quercetin and kaempferol, respectively (Jin et al., 2008; Weisz et al., 2009).

1.2.2 Phytochemicals in the crude extracts

In this study, 95% w/v ethanol was used as a co-solvent in the $scCO_2$ extraction at 25% (w/v) of the liquid CO_2 and as a solvent in the maceration process.

Ethanol is a safe solvent and has high polarity than the $scCO_2$ which can extract polar bioactive compounds from the rice bran such as gamma-oryzanol, glycosides and carotenoids. The phytochemical compounds were shown qualitatively and approximated quantitatively in **Table 13**. Carotenoids which are antioxidants were found in all crude extracts (data not shown). The *C. tinctorius*, *S. bicolor* and *G. max* crude extracts by both processes contained most phytochemicals including alkaloids, carotenoids, glycosides, xanthones and tannins. Hence, the phytochemical constituents were not affected by the extraction processes, but depended on the types of plants. Xanthone and tannins were found in the crude extracts of *C. tinctorius*, *G. max*, *Nelumbo nucifera* Gaertn., *O. sativa*, *S. bicolor* and *Zea may* L.. As known, natural antioxidants are a broad range of phytochemicals including phenolic, nitrogen and carotenoids (Velioglu et al., 1998). The antioxidative activity of the crude extracts which contained these compounds can be anticipated.

1.2.3 Biological activities of the crude extracts

The biological activities including DPPH radical scavenging, lipid peroxidation inhibition, metal ion chelation, tyrosinase inhibition activities and stimulation index of the crude extracts of the ten selected edible plants including bran of *O. sativa* prepared by the two non-heated processes were compared (**Table 14**). Most of plant crude extracts by the $scCO_2$ showed slightly lower activities than those by the ethanolic maceration technique. This might be from the higher content of hydrophilic substance, phenolic compound as shown in the amount of total phenoilic content (TPC).

For DPPH radical scavenging activity, the *C. tinctorius* crude extracts (SC_{50} value of 1.13 ± 0.02 mg/ml) gave the highest DPPH scavenging activity, which higher

Table 13 Comparison of phytochemical compounds of the ten edible plant extracts prepared by the two non-heated methods ($scCO_2$ and ethanolic maceration)

Scientific name	Common name	Part used	Alkaloid		Antraquinone		Carotenoid		Flavonoid		Glycoside						Xanthone		Tannin	
											sucrose		glucose		fructose					
			S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M
Arachis hypogaea L.	peanut	seed	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Carthamus tinctorius L.	safflower	flower	+	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+
Glycine max (L.) Merr.	soybean	seed	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+
Helianthus annuus L.	sunflower	seed	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Linum usitatissimum L.	flax	seed	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Nelumbo nucifera Gaertn.	lotus	seed	-	+	-	-	+	+	-	-	+	+	+	+	-	-	-	-	+	+
Oryza sativa L.	rice	bran from seed	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+
Sesamum indicum L.	sesame	seed	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Sorghum bicolor (L.) Moench	sorghum	seed	+	+	-	-	+	+	-	-	+	+	+	+	-	-	+	+	+	+
Zea may L.	corn	seed	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	-	+

Note: "+" represented presence in the extract

"-" represented absence in the extract

"S" represented supercritical carbon dioxide fluid extraction

"M" represented maceration extraction

Table 14 Comparison of antioxidative, tyrosinase inhibition activities and the stimulation index on human skin fibroblasts (30^{th} passage) of the ten edible plant extracts prepared by the two non-heated methods ($scCO_2$ and ethanolic maceration)

plant scientific name	SC_{50} (mg/ml)		IPC_{50} (mg/ml)		CC_{50} (mg/ml)		IC_{50} (mg/ml)		stimulation index (SI) at 0.1 mg/ml	
	$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration	$scCO_2$	maceration
Arachis hypogaea L.	151.23	52.56	1.23	1.44	ND	ND	ND	ND	1.03	0.76
Carthamus tinctorius L.	1.13	1.66	2.27	1.06	3.58	3.26	19.47	6.64	2.60	2.42
Glycine max (L.) Merr.	7.26	5.77	6.66	10.11	93.05	55.35	ND	ND	2.12	1.77
Helianthus annuus L.	6.36	0.25	0.87	1.79	ND	37.33	ND	5.58	1.45	0.86
Linum usitatissimum L.	28.99	27.65	8.99	27.65	ND	ND	ND	ND	1.13	1.49
Nelumbo nucifera Gaertn.	40.69	6.57	3.19	1.79	10.3	5.05	ND	2.6	0.79	0.94
Oryza sativa L.	6.55	6.79	1.25	1.44	68.23	75.04	25.98	48.06	1.10	1.14
Sesamum indicum L.	24.3	8.32	9.3	0.87	ND	ND	ND	19.29	0.85	0.93
Sorghum bicolor (L.) Moench	2.16	3.64	4.05	3.27	36.48	47.82	3.61	17.24	1.22	1.45
Zea may L.	28.48	22.6	8.48	22.6	7.88	10.41	1.53	3.53	0.73	0.69
vitamin C	0.044		0.07		NA		0.035		1.20	
vitamin E	0.039		0.03		NA		NA		NA	
EDTA	NA		NA		0.15		NA		NA	
kojic acid	NA		NA		NA		0.024		NA	
gamma-linolenic acid	15.49		1.93		1.74		0.77		NA	
linoleic acid	39.82		1.25		14.79		2.55		NA	
oleic acid	55.58		3.51		26.34		7.11		NA	

Note: ND represented not detected

NA represented not appreciable

SC_{50} value (mg/ml) was the concentration of the sample that scavenged 50 % of the DPPH radicals.

IPC_{50} value (mg/ml) was the concentration of the sample that inhibited 50 % of the lipid peroxidation.

CC_{50} value (mg/ml) was the concentration of the sample that chelated 50 % of the metal ion.

IC_{50} value (mg/ml) was the concentration of the sample that inhibited 50 % of the tyrosinase enzyme.

Stimulation index (SI) was the ratio between the percentages of cell proliferation of the treated sample and the control (no treatment).

than those from the *S. bicolor* and *O. sativa* crude extracts by $scCO_2$ of about 1.9 and 5.8 times, respectively, but showed lower activity than the standard vitamin C (SC_{50} value of 0.044 ± 0.004 mg/ml), vitamin E (SC_{50} value of 0.039 ± 0.002 mg/ml) of about 25 and 29 times, respectively. The highest DPPH scavenging activity of the *C. tinctorius* crude extract may be from the high content of phenolic compounds. The significant correlation between DPPH scavenging activity (A_F) and the total phenolic contents (TPC) in this crude extract (r=0.99, p<0.05) was observed. The chemical constituents in this plant have been reported to be flavonoids (Kazuma et al., 2000), lignans (Palter et al., 1972), triterpene alcohols (Akihisa et al., 1996), and polysaccharides (Hirokawa et al., 1997).

For lipid peroxidation inhibition activity, the three crude extracts by $scCO_2$ which gave the highest activity were *H. annuus*, *A. hypogaea* and *O. sativa*, respectively. The *O. sativa* crude extract prepared by $scCO_2$ (IPC_{50} value of 1.25 ± 0.51 mg/ml) showed high lipid peroxidation inhibition activity but lower than vitamin C (IPC_{50} value of 0.07 ± 0.04 mg/ml) and vitamin E (IPC_{50} value of 0.03 ± 0.01 mg/ml) of 17.9 and 41.7 times, respectively. By $scCO_2$, this crude extract gave higher lipid peroxidation inhibition activity than *C. tinctorius* crude extracts (IPC_{50} value of 2.27 ± 0.76 mg/ml) and *S. bicolor* (IPC_{50} value of 4.05 ± 0.69 mg/ml) of about 1.8 and 3.2 times, respectively.

For chelating activity, the three crude extracts by $scCO_2$ which gave the highest activity were *C. tinctorius*, *Z. may* and *S. bicolor*, respectively. The crude extracts from *C. tinctorius* gave the highest chelating activity (CC_{50} value by $scCO_2$ of 3.58 ± 0.17 mg/ml), which was higher than *S. bicolor* (CC_{50} value of 36.48 ± 3.69 mg/ml) and *O. sativa* crude extracts by $scCO_2$ (CC_{50} value of 68.23 ± 8.06 mg/ml) of

about 10.2 and 19 times, respectively, but indicated lower activity than the standard chelating agent, EDTA (CC_{50} value of 0.15 ± 0.04 mg/ml) of about 23 times.

For tyrosinase inhibition activity, the three crude extracts by $scCO_2$ which gave the highest activity were *Z. may*, *S. bicolor* and *C. tinctorius* respectively. The *S. bicolor* crude extract by $scCO_2$ showed the high tyrosinase inhibition activity (IC_{50} value of 3.61 ± 0.82 mg/ml) which was higher than the *C. tinctorius* (IC_{50} value of 19.47 ± 7.35 mg/ml) and *O. sativa* crude extracts by $scCO_2$ (IC_{50} value of 25.98 ± 13.42 mg/ml) of about 5.4 and 7.2 times, respectively, but gave lower activity than the standard vitamin C (IC_{50} value of 0.035 ± 0.009 mg/ml), kojic acid (IC_{50} value of 0.024 ± 0.003 mg/ml) of about 103 and 150 times, respectively.

For human skin fibroblast stimulation activity, the *C. tinctorius* crude extract by $scCO_2$ (SI value of 2.60 ± 0.20) and by maceration (SI value of 2.42 ± 0.67) at 0.1 mg/ml gave the highest stimulation index, which was higher than the standard vitamin C at 0.1 mg/ml (SI value of 1.20 ± 0.08) and *S. bicolor* (SI value of 1.22 ± 0.19 mg/ml) and *O. sativa* crude extracts by $scCO_2$ (SI value of 1.10 ± 0.12 mg/ml) of 2.2, 2.1 and 2.4 times, respectively.

The $scCO_2$ crude extracts from the three plants including *C. tinctorius*, *O. sativa* and *S. bicolor* were selected to prepare the semi-purified fractions because of the highest total unsaturated fatty acid contents (TUC) in comparing to other seven edible plants, since the unsaturated fatty acids, gamma-linolenic acid, linoleic acid and oleic acid, have been proved to have anti-hair loss activity by inhibiting 5α-reductase enzyme in the androgen responsive organs (Liang and Liao, 1992). Although extracts from the $scCO_2$ indicated only slightly lower biological activities than the maceration method, the $scCO_2$ process gave higher unsaturated fatty acid

contents than by the maceration process. Also, the $scCO_2$ method is not only a non-toxic, non-inflammable and inexpensive process but also has high solvating power to extract unsaturated fatty acids.

In addition, the crude extract of *C. tinctorius* showed the highest biological activities, followed by *S. bicolor* and *O. sativa*, respectively. The *C. tinctorius* crude extracts from $scCO_2$ gave the highest DPPH scavenging, chelating and stimulation index, high tyrosinase inhibition activities (in the 3rd rank), moderate lipid peroxidation inhibition (in the 4th rank) and contained the high total unsaturated fatty acids (in the 2nd rank). The crude extract of *O. sativa* which contained the highest total unsaturated fatty acids, showed the moderate biological activities, DPPH scavenging (in the 4th rank), lipid peroxidation inhibition (in the 3rd rank), chelating (in the 4th rank), tyrosinase inhibition activities (in the 4th rank) and stimulation index (in the 6th rank). The *S. bicolor* crude extracts by $scCO_2$ showed the high DPPH scavenging (in the 2nd rank), chelating (in the 3rd rank), tyrosinase inhibition activities (in the 2nd rank) and moderate lipid peroxidation inhibition (in the 6th rank) and stimulation index (in the 4th rank) and contained high amount of total unsaturated fatty acids (in the 3rd rank).

Part 2: Semi-purified fractions preparation from the three selected crude extracts of the three plants and their *in vitro* biological and anti-hair loss activities

2.1 Unsaturated fatty acid and total phenolic contents in semi-purified fractions

Fraction No.3 of *O. sativa* crude extract (OSF3) showed the highest percentage yields (48.23 ± 3.56%, w/w), followed by fraction No.1 of *S. bicolor* (43.67 ± 4.48%, w/w) and fraction No.3 of *C. tinctorius* (37.24 ± 2.72%, w/w) crude

extracts. However, OSF3 contained the highest content of unsaturated fatty acids (gamma-linolenic acid 7.52 ± 1.12%, w/w; linoleic acid 49.25 ±3.67%, w/w; oleic acid 42.17 ± 4.12%, w/w), followed by the crude extract of *O. sativa* (gamma-linolenic acid 5.67 ± 0.52%, w/w; linoleic acid 23.62 ± 2.62%, w/w; oleic acid 27.28 ± 1.22%, w/w) and the fraction No.4 of the *O. sativa* crude extract (gamma-linolenic acid 9.21 ± 0.78%, w/w; linoleic acid 32.07 ±1.31%, w/w; oleic acid 13.71 ± 1.34%, w/w), respectively. The higher contents of unsaturated fatty acids were found in OSF3 than in the crude extracts of about 2 times because the eluent (petroleum ether/ ethyl acetate 8:1) to prepare OSF3 was more non-polar solvent than the salvation system of $scCO_2$ with 25% (w/v) of ethanol.

As known, the $scCO_2$ was the lipophilic solvent that can also extract the colored substances from the plant parts such as safflower and sorghum. So, the *O. sativa*, *C. tinctorius* and *S. bicolor* crude extract were in green, dark orange and dark yellow appearances, respectively, which will be undesirable for cosmetic formulation. Therefore, in order to get rid of the colored substances, these crude extract was semi-purified by column chromatography. The semi-purified fraction of *O. sativa* extract fraction 3 or OSF3, *C. tinctorius* extract fraction 3 and *S. bicolor* extract fraction 1 which had the highest unsaturated fatty acid contents were in pale yellow, pale orange and pale yellow appearances, respectively.

In addition, the crude extract of *C. tinctorius*, fraction No.2 of the *C. tinctorius* crude extract and the crude extract of *S. bicolor* contained high total phenolic contents at 1.36 ± 0.11, 1.25 ± 0.08 and 1.05 ± 0.09 mg of gallic acid equivalent per gram of the extract (GAE mg/g), respectively. These crude extracts

and fractions appeared to give higher phenolic contents because of the slightly polar property of the extraction condition of 25% (w/v) ethanol in $scCO_2$.

2.2 Biological activities of the semi-purified fractions

2.2.1 DPPH radical scavenging assay

The three $scCO_2$ crude extracts and their fractions prepared by column chromatography which gave the highest scavenging activity were the crude extract of *C. tinctorius*, fraction No.2 of *C. tinctorius* crude extract and the crude extract of *S. bicolor* with the SC_{50} values of 1.13 ± 0.12, 1.20 ± 0.25 and 2.16 ± 0.17 mg/ml, respectively (**Table 15**). However, the *C. tinctorius* crude extract which gave the highest DPPH scavenging activity showed lower activity than the standard vitamin C (SC_{50} value of 0.04 ± 0.004 mg/ml), vitamin E (SC_{50} value of 0.04 ± 0.002 mg/ml) of about 28 times, but exhibited higher activity than the standard unsaturated fatty acids, gamma-linolenic acid (SC_{50} value of 15.49 ± 0.02 mg/ml), linoleic acid (SC_{50} value of 39.82 ± 0.05 mg/ml) and oleic acid (SC_{50} value of 55.58 ± 0.9 mg/ml) of about 14, 35 and 49 times, respectively. This indicated that the unsaturated fatty acids in the fractions and the extracts may not be the only bioactive compounds which have the effect on this activity, but also other bioactive compounds such as the total phenolic compounds existing in the crude extracts and fractions. The significant positive linear correlation of free radical scavenging activity (A_F) and the total phenolic content (TPC) of the *C. tinctorius* crude extract (A_F and TPC; $r = 1.00$, $p < 0.01$) was observed, while the positive linear correlation between A_F and the total unsaturated fatty acids content (TUC) of the *C. tinctorius* crude extract was 0.85 (A_F and TUC; $r = 0.85$, $p < 0.05$). Thus, the synergistic effects of both the total phenolic

Table 15 Comparison of the percentage yields, unsaturated fatty acid contents, stimulation index on human skin fibroblasts (30th passage) and percentages of cell viability on DU-145 cells of the fractions from *O. sativa, C. tinctorius* and *S. bicolor* extracts, and their crude extracts

sample	percentage yield (%,w/w)	% unsaturated fatty acid (%, w/w)			TPC (mg GAE/g)	SC_{50} (mg/ml)	IPC_{50} (mg/ml)	CC_{50} (mg/ml)	IC_{50} (mg/ml)	SI (at 0.1 mg/ml)	cell viability (%)
		gamma-linolenic acid	linoleic acid	oleic acid							
O. sativa crude extract	-	5.67	23.62	27.28	0.65	6.55	1.25	68.23	25.98	1.10	102.45
O. sativa fraction No.1	26.44	3.28	16.25	25.32	0.40	7.59	7.92	40.51	39.03	0.99	95.46
O. sativa fraction No.2	21.85	3.08	24.32	24.31	0.39	7.43	11.09	45.63	32.75	0.98	107.55
O. sativa fraction No.3	48.23	7.52	49.25	42.17	0.32	10.91	3.44	53.33	11.90	1.01	99.39
O. sativa fraction No.4	3.38	9.21	32.07	13.71	0.37	8.49	5.09	51.18	22.44	1.03	98.78
C. tinctorius crude extract	-	9.36	4.02	0.65	1.36	1.13	2.27	3.58	19.47	2.60	104.14
C. tinctorius fraction No.1	31.38	0.00	0.15	0.00	0.69	2.50	7.11	7.98	20.11	1.99	102.14
C. tinctorius fraction No.2	22.74	0.00	0.32	0.00	1.25	1.20	8.89	6.58	31.27	1.27	97.45
C. tinctorius fraction No.3	37.24	16.72	15.07	8.40	0.48	5.55	6.29	6.43	19.25	1.59	99.4
C. tinctorius fraction No.4	8.24	10.80	9.86	3.48	0.36	7.54	5.16	9.56	18.56	1.23	99.81
S. bicolor crude extract	-	0.02	5.56	1.78	1.05	2.16	4.05	36.48	3.61	1.22	111.32
S. bicolor fraction No.1	43.67	1.22	11.50	3.26	0.47	6.10	3.56	41.03	13.80	0.99	96.58
S. bicolor fraction No.2	21.23	0.77	9.47	2.74	0.55	5.73	8.33	50.68	13.97	1.12	98.67
S. bicolor fraction No.3	19.54	0.15	4.54	0.87	0.38	8.58	10.34	43.09	5.44	1.04	102.31
S. bicolor fraction No.4	14.63	0.11	2.85	0.93	0.37	7.95	9.90	38.78	12.22	1.01	101.78
gamma-linolenic acid	-	-	-	-		15.49	1.93	1.74	0.77	0.39	19.67
linoleic acid	-	-	-	-		39.82	1.25	14.79	2.55	0.45	37.52
oleic acid	-	-	-	-		55.58	3.51	26.34	7.11	0.99	101.87
finasteride	-	-	-	-		-	-	-	-	1.01	102.86
dutasteride	-	-	-	-		-	-	-	-	1.02	103.27

Note: Total phenolic contents were presented as gallic acid equivalent (GAE) mg/g of the sample.
SC_{50} value (mg/ml) was the concentration of the sample that scavenged 50 % of the DPPH radicals.
IPC_{50} value (mg/ml) was the concentration of the sample that inhibited 50 % of the lipid peroxidation.
CC_{50} value (mg/ml) was the concentration of the sample that chelated 50 % of the metal ion.
IC_{50} value (mg/ml) was the concentration of the sample that inhibited 50 % of the tyrosinase enzyme.
Stimulation index (SI) was the ratio between the percentages of cell proliferation of the treated sample (at 0.1 mg/ml) and the control (no treatment).
Cell viability (%) was the percentages of the viable cell absorbance (at 0.1 mg/ml) divided by the control absorbance (no treatment) on DU-145 cell line.

and unsaturated fatty acid contents had indicated, which may result in the reduction of oxidative stress in the scalp of the alopecia patients (Naziroglu and Kokcam, 2000). The phenoilc compounds may interrupt with the free radical chain and stop further oxidation reaction (Sherwin, 1978).

2.2.2 Lipid peroxidation inhibition assay

The three extracts and fractions which gave the highest lipid peroxidation inhibition activity were the *O. sativa* and *C. tinctorius* crude extract and fraction No.3 of the *O. sativa* crude extract with the IPC_{50} values of 1.25 ± 0.51, 2.27 ± 0.14 and 3.44 ± 0.23 mg/ml, respectively. The *O. sativa* crude extract gave the highest lipid peroxidation inhibition activity, but lower than the standard vitamin C (IPC_{50} value of 0.07 ± 0.009 mg/ml), vitamin E (IPC_{50} value of 0.03 ± 0.005 mg/ml), of about 17.9 and 41.7 times, respectively, while exhibited higher activity than the standard gamma-linolenic acid (IPC_{50} value of 1.93 ± 0.22 mg/ml) and oleic acid (IPC_{50} value of 3.51 ± 0.29 mg/ml) of about 1.5 and 2.8 times, respectively. The *O. sativa* crude extract and OSF3 indicated the positive linear correlation between the total phenolic compound (TPC) contents and the lipid peroxidation inhibition activity (A_L) at $r = 0.93$ ($p<0.05$), showing that the lipid peroxidation inhibition activity (A_L) might be from the high content of the total phenolic compounds (TPC).

2.2.3 Metal ion chelating assay

The three crude extracts and fractions which gave the highest chelating activity were the *C. tinctorius* crude extract, fraction No. 3 of the *C. tinctorius* crude extract and fraction No.2 of the *C. tinctorius* crude extract with the CC_{50} values of 3.58 ± 0.24, 6.43 ± 0.45 and 6.58 ± 0.37 mg/ml, respectively. The *C. tinctorius* crude extract which gave the highest chelating activity, showed lower activity than the

standard EDTA (CC_{50} value of 0.15 ± 0.008 mg/ml) and the standard gamma-linolenic acid (CC_{50} value of 1.74 ± 0.32 mg/ml) of about 23.87 and 2.05 times, but exhibited higher activity than the standard linoleic acid (CC_{50} value of 14.79 ± 1.58 mg/ml) and oleic acid (CC_{50} value of 26.34 ± 2.93 mg/ml) of about 4.13 and 7.36 times, respectively. Although there was the high correlation coefficient (*r*) between the total phenolic compound and chelating activity (A_C) of the *C. tinctorius* crude extract at 0.95 ($p<0.05$), the *C. tinctorius* crude extract showed lower chelating activity than the standards. It may not contain the phenolic compounds which had the complex formation property. The previous study has reported that hydroxytyrosol, gallic acid, caffeic acid and chlorogenic acid were the strong chelator, while vanillic acid, syringic acid and ferulic acid can not chelate the metal ion, showing that not all of the phenolic compounds had the complex formation property (Andjelkovic et al., 2006). However, some chelating activities of these extracts and fractions were observed. In addition, the high Cu level in the occipital region is correlated to the concentrations of free testosterone in the serum which can result in hair loss (Skalnaya and Tkachev, 2010). Thus, the extracts which contained chelators can chelate the excess Cu in the hair follicle and blood, resulting in the decrease of hair loss.

2.2.4 Tyrosinase inhibition assay

The three crude extracts and fractions which gave the highest tyrosinase inhibition activity were the *S. bicolor* crude extract, fraction No. 3 of the *S. bicolor* crude extract and OSF3 with the IC_{50} values of 3.61 ± 0.18, 5.44 ± 1.18 and 11.90 ± 1.47 mg/ml, respectively. The *S. bicolor* crude extract which gave the highest tyrosinase inhibition activity showed lower activity than the standard vitamin C (IC_{50}

value of 0.035 ± 0.009 mg/ml), the standard gamma-linolenic acid (IC_{50} value of 0.77 ± 0.09 mg/ml) and linoleic acid (IC_{50} value of 2.55 ± 0.09 mg/ml) of about 90, 5 and 1.4 times, respectively.

2.2.5 Cytotoxicity on the aged normal human skin fibroblasts

The stimulation index (SI) of the crude extracts and their fractions on aged normal human skin fibroblasts (30^{th} passage) was shown in **Table 15**. At 0.1 mg/ml, all crude extracts and fractions of *O. sativa*, *C. tinctorius* and *S. bicolor*, the standard finasteride and dutasteride and the standard oleic acid showed no cytotoxicity determined by SRB assay, and at 0.01 mg/ml, the standard gamma-linolenic acid and linoleic acid also gave no cytotoxicity. But, at 0.1 mg/ml, the standard gamma-linolenic acid and linoleic acid were toxic to the cells. In fact, linoleic acid has been shown to be toxic to tumor cells with little or no cytotoxic on normal cells at the concentration of more than 0.01 mg/ml (Lu et al., 2010). Thus, these extracts and fractions at 0.1 mg/ml appeared to be safe with no toxicity to be used topically even in the aged human skin fibroblasts. Therefore, the application of this formulation in human scalp skin should be safe for the AGA patient with both young and old human skin fibroblasts.

2.2.6 Cytotoxicity on DU-145 cell line

At 0.1 mg/ml, the crude extracts and fractions of *C. tinctorius*, *O. sativa* and *S. bicolor*, the standard finasteride and dutasteride showed no cytotoxicity on DU-145 cell lines investigated by the SRB assay (**Table 15**). However, at the concentration of 0.1 mg/ml, the standard gamma-linolenic acid and linoleic acid were toxic to the cells. But, gamma-linolenic acid at 0.001 mg/ml (105.45 ± 11.52% of the control) and linoleic acid at 0.01 mg/ml (104.43 ± 12.55% of the control) and oleic

acid at 0.1 mg/ml (101.87 ± 8.47% of the control) exhibited no toxicity to cells. This indicated that the pure unsaturated fatty acids with higher unsaturation were more toxic than those with lower unsaturation since they can induce apoptosis and also necrosis in the cell (Cury-Boaventura et al., 2005). Also, they were more toxic than the crude extracts and semi-purified fractions containing the fatty acids because the phenolic content in the extracts and fractions may have the protective effect against the DNA damage which caused cell death (Greenrod and Fenech, 2003). The crude extracts, their fractions, the standard finasteride, the standard dutasteride and the standard oleic acid at the highest concentration of 0.1 mg/ml and the standard gamma-linolenic acid and linoleic acid at the highest concentration of 0.001 and 0.01 mg/ml, respectively, were used in the 5α-reductase inhibition experiment.

2.3 The 5α-reductase type 1 inhibition assay

The type 1, 5α-reductase, is the main cause of hair loss, predominated in human scalp skin especially in dermal papilla (Thiboutot et al., 1995). In several studies, the DU-145 human androgen insensitive prostate adenocarcinoma cell line which has the type 1, 5α-reductase has been used for the 5α-reductase type 1 inhibition assay (Délos et al., 1994; Handratta et al., 2005). The OSF3 at 0.1 mg/ml showed the highest 5α-reductase type 1 inhibition activity on DU-145 cell line at 93.33 ± 10.93 % of the control which was higher than the standard finasteride (57.07 ± 6.52%) and dutasteride (76.56 ± 4.76%) of about 1.64 and 1.22 times, respectively (**Figure 13)**. The percentages inhibition on 5α-reductase (type 1) of gamma-linolenic acid (0.001 mg/ml) and oleic acid (0.1 mg/ml) were only at 38.72 ± 5.36 and 35.69 ± 5.01%, respectively, which was lower than OSF3 at about 2.41 and 2.61 times, respectively. However, the standard linoleic acid (0.01 mg/ml) showed similar

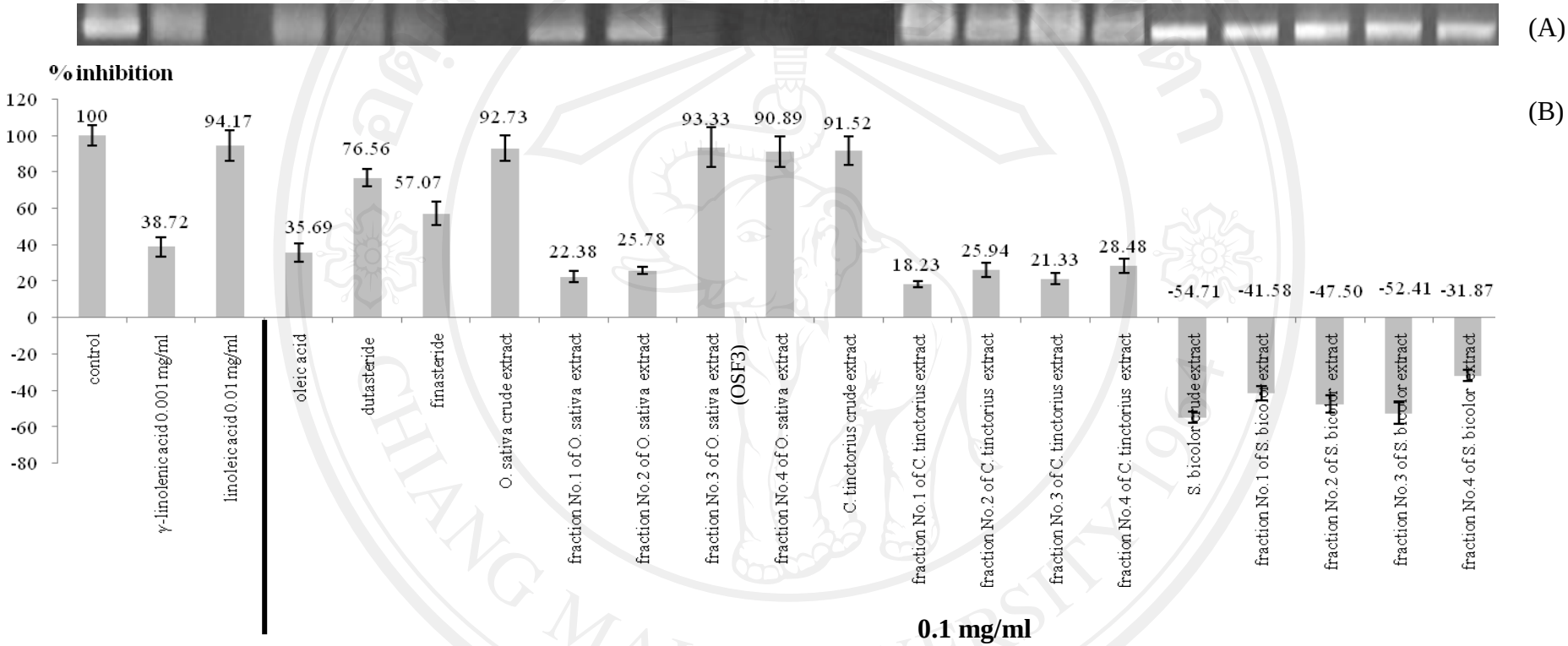


Figure 13 The 5α-reductase (type 1) inhibition on DU-145 cells at 0.1 mg/ml of *O. sativa*, *C. tinctorius* and *S. bicolor* crude extracts and their fractions in comparing to the standards finasteride (0.1 mg/ml), dutasteride (0.1 mg/ml), standard unsaturated fatty acids, gamma-linolenic acid (0.001 mg/ml), linoleic acid (0.01 mg/ml) and oleic acid (0.1 mg/ml). (A) agarose gel electrophoresis of dsDNA of 5α-reductase (type 1) enzyme after the inhibition of the samples and (B) the percentages of 5α-reductase (type 1) inhibition. % inhibition = [(control-sample)/control] x 100

inhibition activity (94.17 ± 8.27%) to the OSF3. Moreover, the inhibition activity of the standard linoleic acid was also dose-dependent which was in the same trend as OSF3. When linoleic acid was increased from 0.0001, 0.001 and 0.01 mg/ml, it gave an increasing inhibition at 51.23 ± 5.38, 61.58 ± 7.30 and 94.17 ± 8.27%, respectively ($y= 4047x+ 54.01$, $r^2 = 0.977$). Thus, linoleic acid appeared to play the main role for the inhibition of the 5α-reductase inhibition activity. Besides linoleic acid, other bioactives existing in the fractions, such as ferulic acid, vanillic acid, gamma-oryzanol and phytic acid (Das et al., 1998) in the OSF3 which had well-known antioxidative activities may be synergistic for this activity. For OSF3, the significant positive linear correlation between the total unsaturated fatty acids and 5α-reductase inhibition activity (TUC and 5AR; $r = 1.00$, $p<0.01$) and linoleic acid contents and 5AR (LN and 5AR; $r = 1.00$, $p<0.01$) were observed. Moreover, the 5α-reductase inhibition activity of the OSF3 also correlated with the antioxidative activities including, free radical scavenging activity (A_F and 5AR; $r = 0.98$, $p<0.05$), lipid peroxidation inhibition activity (A_L and 5AR; $r = 0.95$, $p<0.05$) and chelating activity (A_C and 5AR; $r = 0.89$, $p<0.05$) and its phenolic contents (TPC and 5AR; $r = 0.97$, $p<0.05$) (**Table 16**). This indicated that the 5α-reductase inhibition activity of the OSF3 was related to antioxidant activities and the phenolic contents. For DPPH radical scavenging activity, inadequate antioxidant protection or excess production of reactive oxygen species (ROS) creates a condition known as oxidative stress, which can cause hair loss (Löntz et al., 1995; Miyachi, 1995). In fact, the relationship between the free radical scavenging activity and the anti- androgenic alopecia activity can be anticipated, since there is a balance between the oxidative damage and antioxidant protection in normal aerobic cells. This study has shown the correlation

Table 16 The correlation matrix (Pearson's correlation coefficients) of the unsaturated fatty acid contents, total phenolic contents, stimulation index on human skin fibroblasts (passage 30th) and the 5α reductase inhibition activity of *O. sativa* crude extract, OSF3 and *C. tinctorius* crude extract (at 95% confidence interval)

extract/ fraction	variable	GLA	LN	OL	TUC	TPC	A_F	A_L	A_C	A_T	SI	5AR
O. sativa crude extract	GLA	-										
	LN	0.88	-									
	OL	0.82	0.82	-								
	TUC	0.78	0.98	0.91	-							
	TPC	0.94	0.93	0.93	0.95	-						
	A_F	0.91	0.89	0.91	0.91	0.96	-					
	A_L	0.91	0.87	0.89	0.92	0.93	0.84	-				
	A_C	0.93	0.88	0.91	0.89	0.95	0.78	0.88	-			
	A_T	0.90	0.91	0.89	0.88	0.92	0.83	0.89	0.79	-		
	SI	0.91	0.87	0.93	0.85	0.82	0.91	0.93	0.91	0.90	-	
	5AR	0.95	0.99*	0.97	0.99*	0.95	0.95	0.93	0.91	0.89	0.91	-
fraction No.3 of *O. sativa* crude extract (OSF3)	GLA	-										
	LN	0.87	-									
	OL	0.82	0.82	-								
	TUC	0.76	0.99*	0.91	-							
	TPC	0.89	0.93	0.93	0.95	-						
	A_F	0.93	0.89	0.91	0.91	0.97	-					
	A_L	0.94	0.87	0.89	0.92	0.93	0.84	-				
	A_C	0.89	0.88	0.91	0.89	0.95	0.78	0.88	-			
	A_T	0.90	0.91	0.89	0.88	0.92	0.83	0.89	0.79	-		
	SI	0.90	0.87	0.93	0.85	0.82	0.91	0.93	0.91	0.90	-	
	5AR	0.96	1.00**	0.95	1.00**	0.97	0.98	0.95	0.90	0.93	0.93	-
C. tinctorius crude extract	GLA	-										
	LN	0.84	-									
	OL	0.79	0.80	-								
	TUC	0.89	0.85	0.76	-							
	TPC	0.87	0.82	0.73	0.85	-						
	A_F	0.78	0.83	0.89	0.85	1.00**	-					
	A_L	0.87	0.78	0.71	0.84	0.98	0.84	-				
	A_C	0.67	0.74	0.87	0.73	0.95	0.67	0.78	-			
	A_T	0.80	0.71	0.89	0.88	0.88	0.83	0.89	0.79	-		
	SI	0.88	0.85	0.88	0.89	0.91	0.94	0.93	0.89	0.79	-	
	5AR	0.89	0.95	0.90	0.93	0.98	0.91	0.90	0.83	0.89	0.92	-

Note: GLA: gamma-linolenic acid content; LN: linoleic acid content, OL: linoleic acid content, TUC: total unsaturated fatty acid content, TPC: total phenolic content, A_F: free radical scavenging activity, A_L: lipid peroxidation inhibition activity, A_C: chelating activity, A_T: tyrosinase inhibition activity, SI: stimulation index, 5AR: 5α reductase inhibition activity

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

of A_F and the 5α-reductase inhibition activity (5AR) of the *C. tinctorius* crude extract (A_F and 5AR; $r = 0.91$, $p < 0.05$)**.** For lipid peroxidation activity, lipid peroxidation can be caused by free radicals which can initiate the free radical chain reaction on the cell membrane in hair dermal papilla. Thus, these crude extracts and fractions may inhibit free radicals which initiated lipid peroxidation reaction in cell membrane involved hair dermal papilla, resulting in cell damage and hair shedding (Das, 1991). Moreover, the metal ion chelating activity may be able to reduce the excess of Cu level in the occipital area and also in the blood circulation of the androgenic alopecia patients (Skalnaya and Tkachev, 2010). The moderate tyrosinase inhibition activity of these fractions and crude extracts might be useful for the 5α-reductase inhibition activity by binding with the enzyme that can have the synergistic effect with other compounds.

The *O. sativa* and *C. tinctorius* crude extracts and fraction No.4 of the *O. sativa* crude extract also exhibited marked 5AR inhibition activity at 92.73 ± 6.91, 91.52 ± 7.69 and 90.89 ± 8.5 % of the control, respectively, while the *S. bicolor* crude extract and their fractions showed the negative result which stimulated the production of 5α-reductase enzyme. Similarly, the fractions of *C. tinctorius* crude extract exhibited low 5AR inhibition activity. The significant positive linear correlation between TUC and 5AR ($r = 0.99$, $p<0.05$) and LN and 5AR ($r = 0.99$, $p<0.05$) of the *O. sativa* crude extracts were shown. The *C. tinctorius* crude extracts showed positive correlation between TUC and 5AR and between LN and 5AR at linear relationship of $r = 0.93$ and 0.95, $p<0.05$, respectively. The unsaturated fatty acids may have less effect on the 5α-reductase inhibition activity than TPC with the correlation at (TPC and 5AR, $r = 0.98$, $p<0.05$) in the *C. tinctorius* crude extracts.

Part 3: Development of blank neutral niosomes and the niosomes loaded with *O. sativa* crude extract by chloroform film method and $scCO_2$

3.1 The maximum loading of the crude extracts in niosomes

There were yellow separate layers on the surface of the niosomal dispersion when the loaded rice bran extract was more than 0.25 % w/w. Moreover, the membrane microviscosity also can not be performed at this concentration since peak fluctuation was observed. Hence, the maximum loading of the crude extract in niosomes was 0.25 % w/w. This low loading concentration of the extract in niosomes might be from the small particle size (less than 100 nm) and the unilamellar structure of niosomes prepared from the high power of the sonication process (Feitosa et al., 2006). **Figure 14** showed the TEM image of the unilamellar niosomal structures (prepared by $scCO_2$) loaded with 0.25 % w/w of the crude extract prepared by $scCO_2$.

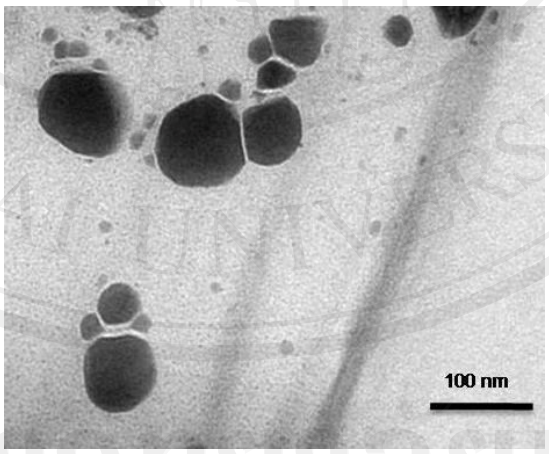


Figure 14 The TEM image of niosomes (Tween 61/cholesterol at 1:1 molar ratio) loaded with the rice bran crude extract (prepared by $scCO_2$) at 0.25% (w/w), indicating the particle size of about 100 nm

3.2 Particle size and morphology determination

All niosomes prepared by the two methods (chloroform film method and $scCO_2$) of the loaded and not loaded with the crude extract exhibited no significant particle size difference at the average size of 60.34 ± 30.91 nm (p<0.05) with unilamellar structures (**Table 17**). **Figures 15A** and **15B** showed the FF-TEM of niosomes loaded with 0.25 % w/w of the extract (prepared by $scCO_2$) prepared by $scCO_2$ and the chloroform film method, respectively.

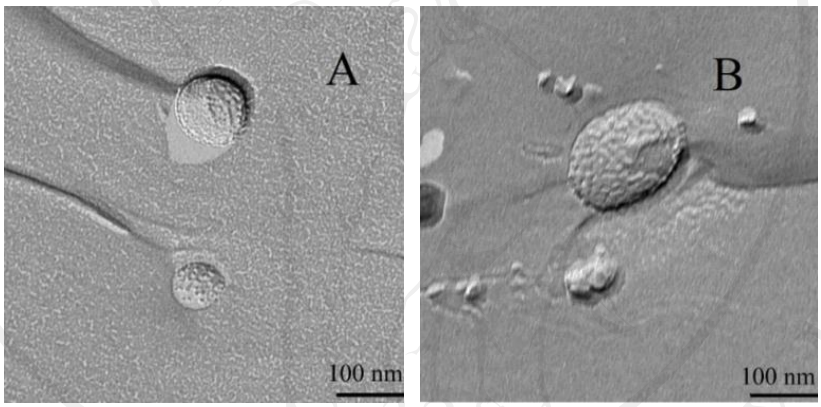


Figure 15 The freezed fracture (FF) TEM images of niosomes (Tween 61/cholesterol at 1:1 molar ratio) loaded with the rice bran extract (by $scCO_2$) at 0.25% (w/w) prepared by (A) $scCO_2$ technique and (B) conventional chloroform film method

3.3 Transition temperature analysis of niosomes

The phase transition temperatures (Tc) of all niosomes loaded and not loaded (blank) with the extracts at all concentrations prepared by both methods did not show any significant difference (p<0.05) at about 80^{o}C (**Table 17**). The extracts prepared by the $scCO_2$ and ethanolic maceration gave the Tc at 71.32 ± 2.34^{o}C and 77.54 ±

Table 17 Comparison of the vesicular sizes and transition temperatures of blank niosomes and niosomes loaded with the rice bran extract at various concentrations

sample	rice bran extract concentration (% w/w)	vesicular sizes (nm)		transition temperature (^oC)	
		$scCO_2$ method	chloroform film method	$scCO_2$ method	chloroform film method
blank niosomes	-	50.91 ± 28.68	50.54 ± 30.77	80.20 ± 3.87	76.61 ± 2.36
niosomes loaded	0.06	58.75 ± 28.94	56.94 ± 29.45	79.44 ± 2.18	77.39 ± 2.39
with the rice bran crude extract	0.12	75.67 ± 36.52	51.75 ± 31.05	77.87 ± 3.79	78.09 ± 4.63
(from $scCO_2$ extraction)	0.25	73.68 ± 30.97	56.65 ± 33.13	77.21 ± 1.27	79.27 ± 4.33
niosomes loaded	0.06	44.86 ± 26.25	46.66 ± 27.16	76.97 ± 2.54	79.93 ± 2.64
with the rice bran crude extract	0.12	67.35 ± 32.05	59.18 ± 29.62	77.30 ± 1.87	80.63 ± 2.80
(from ethanolic maceration)	0.25	66.65 ± 34.14	64.17 ± 32.45	76.71 ± 1.47	77.25 ± 0.74

Note: - Niosomes (Tween 61/cholesterol at 1:1 molar ratio) were prepared by $scCO_2$ and the conventional chloroform film methods.

- Values represent mean ± S.D. (n=3)

1.59^{o}C, respectively. Thus, Tc of all niosomes loaded with the crude extract did not significantly change to lower temperature in comparing to the blank niosomes and the crude extracts. This might be from the low loaded extract concentration in niosomes. Thus, the crude extract at the concentration lower than 0.25 % w/w did not affect the Tc of the niosomes. It has also been reported that unilamellar vesicles exhibited a broad small peak, whereas the multilamellar vesicles (MLVs) gave a large sharp peak of Tc (Guinedi et al., 2005). The Tc peak shapes of the niosomal formulations were broad and small (data not shown). This has confirmed the unilamellar structure of the niosomes.

3.4 Microviscosity of niosomal membrane

The similar DPH graph trend of the niosomes by the $scCO_2$ and the conventional chloroform film method both loaded and not loaded with the extracts (by $scCO_2$ and ethanolic maceration) at the concentrations of 0.06, 0.12 and 0.25 % w/w was observed. **Figure 16** showed the DPH graphs indicating the relationship between the fluorescence polarization (P) and the temperature (^{o}C) of various niosomal formulations prepared by $scCO_2$, and loaded with the extract by $scCO_2$ (A) and ethanolic maceration (B) at 0.06, 0.12, 0.25, 1.00 and 3.00 % w/w. The fluorescence polarization in all niosomes indicated a gradual decrease with the continuous phase transition from gel to liquid crystalline phase of the niosomal membrane when the temperature was increased. This has agreed with the previous study that membrane microviscosity of niosomes slightly decreased with increased temperatures (Manosroi et al., 2008a). As known, the high fluorescence polarization (P) indicated high microviscosity of the niosomal membrane (Manosroi et al., 2003). Niosomes loaded with high concentration of the crude extract (0.25 % w/w) showed lower fluorescence

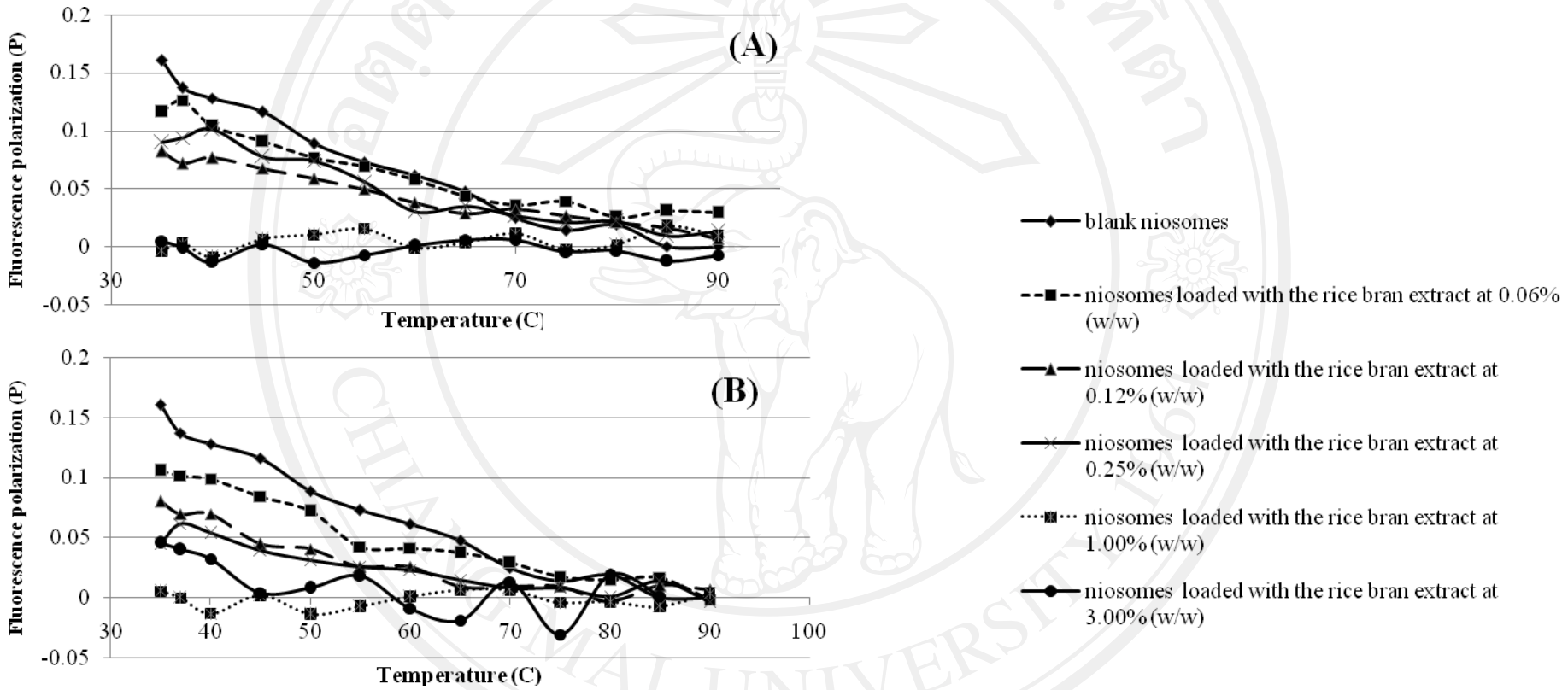


Figure 16 The relationship between the temperatures (^{o}C) and the fluorescence polarization (P) of blank niosomes composed of Tween 61/ cholesterol at 1:1 molar ratio (not loaded with the rice bran extract) and niosomes loaded with the extract at 0.06 to 3.00% (w/w) prepared by $scCO_2$ technique, (A) the crude extract prepared by $scCO_2$ and (B) the crude extract prepared by ethanolic maceration.

polarization than those loaded with low concentration of the crude extract (0.06 % w/w). This has demonstrated that higher extract concentrations gave lower microviscosity with less rigid niosomal membrane than lower extract concentrations. Also, the fluctuated peaks of niosomes when loaded with the crude extract at the concentration higher than 0.25 % w/w were observed (**Figure 16**). This may be due to the interference of the loaded extract on the DPH probe in the lipophillic region of the niosomal membrane (Manosroi et al., 2003**).** Moreover, the unloaded extracts may also affect the ability of the probe to attach on the niosomal membrane, thereby concealing the fluorescences.

Part 4: Development of the blank cationic niosomes and the cationic niosomes loaded with OSF3 containing unsaturated fatty acids by $scCO_2$ technique

4.1 Characteristics of various blank neutral and cationic niosomes

The mean sizes, zeta potential and % cell viability on human skin fibroblast of blank neutral and cationic niosomes were shown in **Table 18**. After 3 months at different storage temperatures, the neutral niosomes (20mM of Tween 61:cholesterol) gave the mean particle size at 96.70±0.22 nm with the negative value of zeta potential of -37.03±1.80 mV indicating the physical stability which agreed with the previous study (Manosroi et al., 2010). For blank cationic niosomes prepared from CTAB and CPC, they exhibited the larger size (at 1:1:0.05 molar ratios) and lower zeta potential values (at 1:1:0.05 and 1:1:0.25 molar ratios) outside the ± 30 mV range demonstrating the physical stability of the dispersion (Gibson et al., 2009). CPC at 1:1:0.5 molar ratio indicated high toxicity on human skin fibroblast with the % cell viability of 68.15±6.63. The BZKC niosomal dispersion at 1:1:0.05 and 1:1:0.25

Table 18 Vesicular sizes (nm), zeta potential (mV) and percentages of cell viability on human skin fibroblast (passage 27th) of blank cationic niosomes prepared from various cationic surfactants at initial and after stored at 25±2 °C for 3 months

formulation		size (nm)		zeta potential (mV)		% cell viability
Tween 61/cholesterol/cationic surfactant	molar ratio	at initial	after 3 months	at initial	after 3 months	
Tween 61/chol/CTAB	1:1:0.05	107.40 ± 1.57	1755.00 ± 18.33	31.23 ± 1.04	19.40 ± 0.79	99.24 ± 10.32
	1:1:0.25	104.47 ± 1.12	108.40 ± 1.04	53.57 ± 1.12	14.00 ± 2.17	90.15 ± 8.67
	1:1:0.5	107.97 ± 1.89	112.27 ± 0.25	69.80 ± 3.61	52.60 ± 2.33	82.92 ± 7.53
Tween 61/chol/CPC	1:1:0.05	107.13 ± 1.69	855.73 ± 1.42	40.93 ± 1.01	18.93 ± 0.85	86.45 ± 8.21
	1:1:0.25	118.13 ± 0.32	118.67 ± 1.46	69.00 ± 4.90	17.37 ± 5.89	78.81 ± 7.87
	1:1:0.5	109.23 ± 1.52	115.60 ± 0.79	70.13 ± 1.97	47.73 ± 0.64	68.15 ± 6.63
Tween 61/chol/DDAB	1:1:0.05	139.50 ± 1.32	separation layer	14.30 ± 0.72	separation layer	84.66 ± 8.28
	1:1:0.25	120.53 ± 3.74	931.17 ± 1.29	49.00 ± 5.83	24.77 ± 2.70	96.47 ± 9.26
	1:1:0.5	112.87 ± 1.67	314.17 ± 1.07	59.20 ± 8.49	27.50 ± 1.11	87.07 ± 9.25
Tween 61/chol/ST	1:1:0.05	117.70 ± 1.73	separation layer	30.45 ± 0.32	separation layer	92.11 ± 6.78
	1:1:0.25	675.33 ± 36.71	separation layer	45.98 ± 0.89	separation layer	98.68 ± 8.61
	1:1:0.5	234.43 ± 7.23	separation layer	37.73 ± 0.85	separation layer	88.65 ± 8.93
Tween 61/chol/BZKC	1:1:0.05	111.00 ± 0.53	107.20 ± 1.85	50.10 ± 3.77	17.87 ± 1.17	98.41 ± 8.72
	1:1:0.25	115.07 ± 1.15	108.37 ± 0.21	44.13 ± 1.25	21.07 ± 0.49	123.91 ± 9.58
	1:1:0.5	106.17 ± 2.94	110.10 ± 0.82	58.27 ± 4.64	43.50 ± 1.30	102.76 ± 8.35
Tween 61/chol/BZT	1:1:0.05	142.93 ± 6.69	separation layer	-0.43 ± 0.02	separation layer	81.83 ± 2.59
	1:1:0.25	103.67 ± 2.58	115.57 ± 1.01	17.70 ± 0.95	7.08 ± 1.13	88.52 ± 7.54
	1:1:0.5	98.66 ± 1.40	100.10 ± 1.01	29.30 ± 1.85	13.40 ± 5.60	91.12 ± 8.99
Tween 61/chol	1:1	96.70 ± 0.22	94.76 ± 0.71	-37.03 ± 1.80	-32.53 ± 0.45	81.10 ± 6.92

Note: Each value represented mean±S.D. of three experiments.

Cationic niosomes of Tween 61/cholesterol/ cationic surfactants at various molar ratios. Cationic surfactants composed of CTAB (cetyl trimethyl ammonium bromide), CPC (cetylpyridinium chloride), DDAB (didecyl dimethyl ammonium bromide), ST (stearylamine), BZKC (benzalkonium chloride) and BZT (benzethonium chloride).

molar ratios, as well as the DDAB and the BZT niosomal dispersion at 1:1:0.25 and 1:1:0.5 molar ratios gave low zeta potential values outside the physical stable range. The DDAB and BZT niosomes at 1:1:0.05 molar ratio and the SA niosomes at 1:1:0.05, 1:1:0.25 and 1:1:0.5 molar ratios indicated separation layers. Thus, the blank cationic niosomes prepared from CTAB and BZKC (at 1:1:0.5 molar ratio) and neutral niosomes (at 1:1 molar ratio) which exhibited physical stability and low cytotoxicity were selected to load with OSF3.

4.2 Physicochemical stability of niosomes loaded with OSF3

The OSF3 was selected to loaded in niosomes due to their high unsaturated fatty acid contents, antioxidative activities and *in vitro* 5α reductase inhibition (Ruksiriwanich et al., 2011). The 20 mM of neutral niosomes and cationic niosomes (CTAB and BZKC) at 1:1:0.5 molar ratio were selected to load with the OSF3 due to their size consistency and proper zeta potential as well as low toxicity on normal human skin fibroblast (30^{th} passage).

4.2.1 The maximum loading of OSF3 in niosomes

When the loading concentration was more than 2.00% (w/v) of OSF3, yellow separate layers on the surface of both neutral and cationic niosomal dispersion were observed. The maximum loading of OSF3 in cationic niosomes was 2.00% (w/v) which was higher than the *O. sativa* bran crude extract in the previous study (0.25%, w/v). This was not only due to the different $scCO_2$ apparatus, but also the different conditions and extracts from the previous study (Manosroi et al., 2010).

4.2.2 Size and zeta potential of niosomes loaded with OSF3

Size and zeta potential of the loaded niosomes were presented in **Table 19**. Sizes of OSF3 loaded niosomes were slightly larger than the blank

niosomes. Size of the loaded neutral niosomes (280 nm) was about 2 times larger than the loaded cationic niosomes (CTAB and BZKC) (170 nm). The smaller size of the loaded cationic niosomes than the loaded neutral niosomes may be from the interaction between the negative charges of the unsaturated fatty acid in OSF3 and the positive charge of the cationic niosomes, thereby condensing the vesicular sizes. The neutral niosomes which had negative charge from their zeta potential values may repulse the negative charges of the unsaturated fatty acids resulting the larger vesicular sizes (Li and Mitra, 1996). The negative charges of the carboxyl group in the unsaturated fatty acids of OSF3 (Leaf et al., 2003) may also affect the zeta potential value of both blank neutral and cationic niosomes (CTAB and BZKC) (-37.03, 69.80 and 58.27 mV) to be more negative (-50.33, 48.00 and 31.53 mV) when loaded with OSF3. The blank neutral niosomes were stable for 3 months at 4, 25 and 45°C. But, when the neutral and BZKC niosomes loaded with OSF3, some separation layers were observed when stored at 45°C for 2 months. Therefore, the loaded CTAB niosomes (1:1: 0.5 molar ratio) was selected for further transfollicular study since it gave the stable dispersion with no precipitation or color change with the particle size of 156.07, 164.40, 174.59 nm and zeta potential values of 54.37, 56.70. 55.43 mV when stored at 45°C for 1, 2 and 3 months, respectively.

4.2.3 Chemical stability of unsaturated fatty acids in OSF3 loaded in niosomes

The percentage (%) remaining of the unsaturated fatty acids (gamma-linolenic acid, linoleic acid and oleic acid) in the OSF3 solution and OSF3 niosomes were presented in **Figure 17.** Temperatures appeared to affect the stability of the

Table 19 Vesicular sizes (nm), zeta potential (mV) and percentages of cell viability on human skin fibroblast (passage 27th) of cationic and neutral niosomes loaded with OSF3 at initial and after stored for 3 months at 4, 25 and 45 °C

formulations	temperature (°C)	sizes (nm)		zeta potential (mV)		% cell viability
		at initial	after 3 months	at initial	after 3 months	
OSF3 loaded in CTAB niosomes (Tw61/chol/CTAB)	4	167.43 ± 3.49	192.47 ± 4.23	48.00 ± 1.31	44.65 ± 2.21	81.45 ± 4.84
	25	167.43 ± 3.49	180.61 ± 5.29	48.00 ± 1.31	48.18 ± 3.67	
	45	167.43 ± 3.49	174.59 ± 10.72	48.00 ± 1.31	55.43 ± 3.52	
OSF3 loaded in BZKC niosomes (Tw61/chol/BZKC)	4	169.17 ± 6.17	203.22 ± 7.32	31.53 ± 0.87	27.44 ± 1.02	93.43 ± 6.38
	25	169.17 ± 6.17	211.65 ± 8.27	31.53 ± 0.87	30.54 ± 1.97	
	45	169.17 ± 6.17	separation layer	31.53 ± 0.87	separation layer	
OSF3 loaded in neutral niosomes (Tw61/chol)	4	273.70 ± 10.82	340.30 15.74	-50.33 ± 2.05	-45.12 ± 1.77	79.69 ± 4.15
	25	273.70 ± 10.82	289.00 19.12	-50.33 ± 2.05	-35.53 ± 1.94	
	45	273.70 ± 10.82	separation layer	-50.33 ± 2.05	separation layer	
vitamin C		-	-	-	-	120.14 ± 8.23

Note: Each value represented mean±S.D. of three experiments.

Cationic niosomes were prepared by Tween 61/cholesterol/cationic surfactants, CTAB (cetyl trimethyl ammonium bromide), and BZKC (benzalkonium chloride).

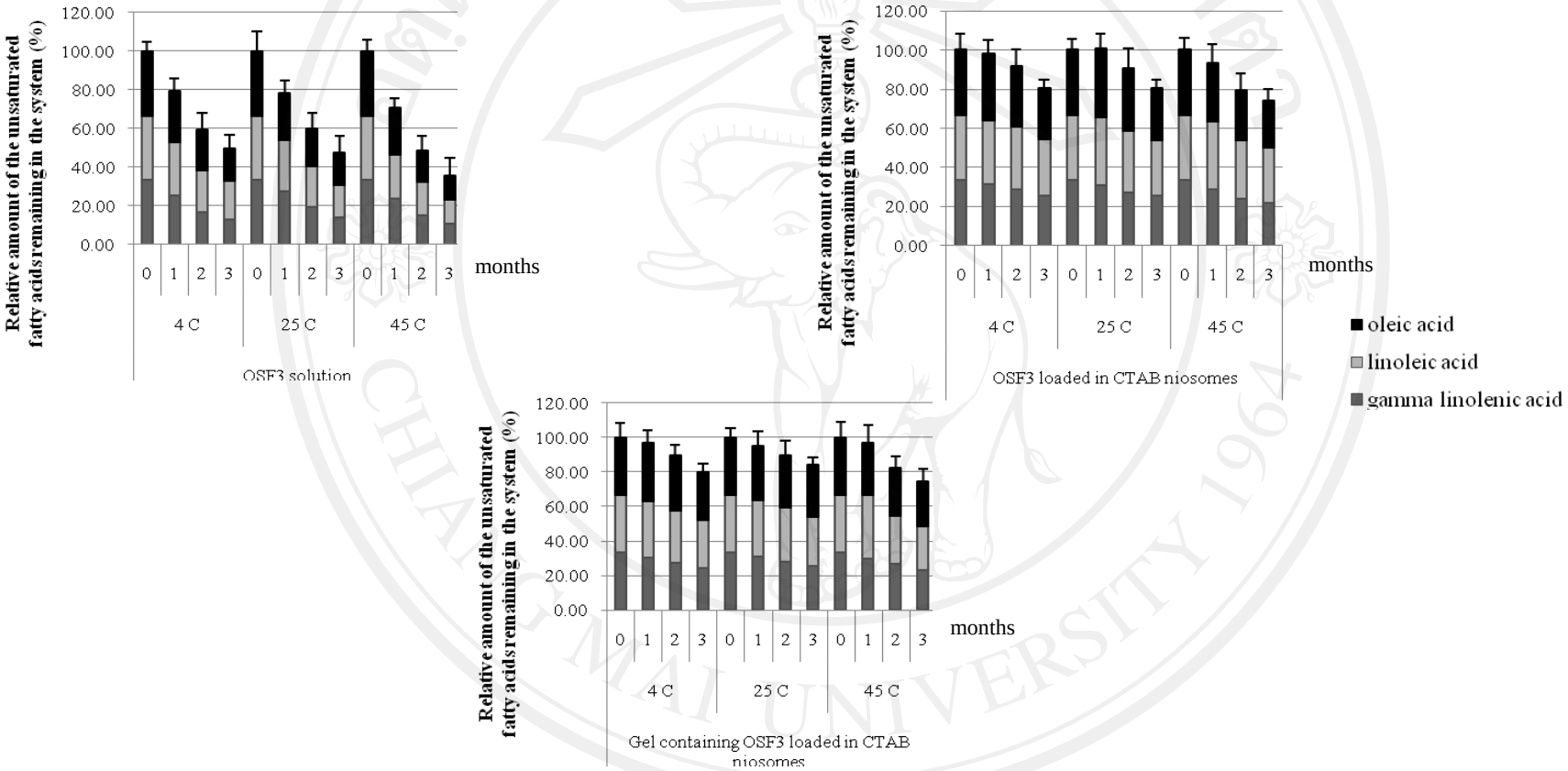


Figure 17 Chemical stability of the unsaturated fatty acids (gamma-linolenic acid, linoleic acid and oleic acid) and the total unsaturated fatty acids in various formulations after stored at 4, 25 and 45 °C for 3 months

unsaturated fatty acids in OSF3. The unsaturated fatty acid contents in both systems at 45°C were the lowest in comparing to at 4 and 25°C. The storage times and types of niosomes also affected the contents of the unsaturated fatty acids. At 25°C for 3 months, neutral niosomes gave the total unsaturated fatty acid remaining of less than 60% significantly different (p<0.05) from other niosomal formulations (data not shown). At 45°C, this formulation gave separation layer. Although the loaded BZKC niosomes stored at 4 and 25°C gave the high remaining total unsaturated fatty acids of about 75%, it gave separation layer at 45°C (data not shown). The loaded CTAB niosomes (Tween 61/cholesterol/CTAB) at 1:1:0.5 molar ratio gave no separation layer with the total unsaturated fatty acids contents of more than 73% when kept at 4, 25 and 45°C for 3 months. The loaded CTAB niosomes exhibited higher content of all unsaturated fatty acids than the OSF3 solution at 4, 25 and 45°C for 3 months. This may be due to the stability enhancement and the protection of the degradation of the unsaturated fatty acids of niosomes. Moreover, the cationic surfactant in niosomes can protect the negatively charged unsaturated fatty acids better than the neutral niosomes owing to the charge interaction (Meng et al., 2008).

At all storage temperatures, both systems (OSF3 solution and OSF3 niosomes) gave the lowest contents of gamma-linolenic acid. The numbers of the double bonds in gamma-linolenic acid (18:3 *n*-6) were more than those in linoleic acid (18:2, *n*-6) and oleic acid (18:1, *n*-9) which can be more readily oxidized to ketone or aldehyde causing rancidity. It has been reported that the high number of double bonds in the fatty acid chains increases the susceptibility to oxidation (Abuzaytoun and Shahidi, 2006). In fact, a double bond consists of two components including a strong symmetry sigma (σ) bond and a pi (π) bond. The π-bond has lower energy and is

responsible for the greater reactivity of the unsaturated compounds (Merrill et al., 2008). Thus, the tendency of the unsaturated fatty acids to undergo oxidation is related to the number of the double bonds in the fatty acid molecule, resulting in the lowest contents of gamma-linoleic acid in all systems.

4.3 Physico-chemical characteristics of CTAB cationic niosomes loaded with OSF3 prepared by supercritical carbon dioxide fluid ($scCO_2$)

4.3.1 Appearance of the formulations and the entrapment efficiency of OSF3 loaded in CTAB cationic niosomes

The CTAB niosomes (Tween 61/cholesterol/CTAB at molar ratio of 1:1:0.5) was selected to entrap various concentrations of OSF3. The blank formulation showed the most clear translucent appearance. More OSF3 loaded in the niosomes can result with the cloudier white niosomal formulations. The CTAB niosomes with the maximum loading of OSF3 at 2.0% (w/v) showed the white milky niosomal appearance. This may be from the oil droplets which can not be loaded inside the vesicles, suspended around the vesicles and emulsified by the surfactant. After ultracentrifugation, there were 2 groups including the no-oil excess group (blank and the 0.1% OSF3 niosomes) and the oil excess group (the 0.5, 1.0 and 2.0% OSF3 niosomes). The no-oil excess group was separated into 2 layers with the up layer and the precipitate gel. The oil excess group was separated into 3 layers including oil layer, mid layer and the precipitate gel. The precipitate gel was the accumulation of the niosome vesicles which were ultracentrifuged. The oil layer in the oil excess group might be from the suspended excess OSF3.

The 0.5% OSF3 niosomes from the oil excess group was selected to investigate for the entrapment efficiency by the ultracentrifugation method because of

its lowest concentration of OSF3 in the oil excess group. The entrapment efficiency of the 0.5% OSF3 niosomes was 86.22±1.43% resulting in the observed excess oil layer of probably 13% after ultracentrifugation. Moreover, the higher maximum loading (2.0% w/v) of OSF3 in niosomes than that in our previous study (0.25%) might be from the higher pressure of $scCO_2$ (250 bar) than that used in the previous study (200 bar). Moreover, not only the high pressure of 250 bar of $scCO_2$, but also the oil-soluble property of Tween 61 (Goyan et al., 1961) can suspend the excess oil droplets of up to 2.0% OSF3 without giving the separation layer, as well.

4.3.2 Particle sizes and niosomal morphology

Table 20 showed the vesicular sizes of all formulations (blank niosome, 0.1, 0.5, 1.0 and 2.0% w/v of OSF3 loaded in niosomes) before and after ultracentrifugation. The sizes of the niosomes were slightly larger when loaded with OSF3 at about 120 to 220 nm. In fact, the small size of the niosomes can increase the physical stability of the formulation according to the Stokes' law indicating that the velocity of the droplet is proportional to the square of its radius. Therefore, the stability of the niosomes can be enhanced by reducing the droplet sizes (Fustier et al., 2010). The expansion of the membrane may be due to the intervention of the anionic carboxyl group of the unsaturated fatty acids containing in OSF3 between the cationic polar group of the cationic lipid composed in niosomes. Moreover, Tween 61 can solubilize both the lipophilic OSF3 and cholesterol at the niosomal membrane resulting in the intervention of Tween 61, cholesterol, CTAB and OSF3 at the niosomal membrane (Deo and Somasundaran, 2003). After ultracentrifugation, sizes of all niosomes (blank, 0.1 and 0.5% OSF3 niosomes) increased to 200 nm except the

Table 20 Vesicular sizes, zeta potential values and transition temperatures of CTAB cationic niosomes loaded with OSF3 at various concentrations before and after centrifugation

formulation	vesicular sizes (nm)		zeta potential (mV)		transition temperature (°C)	
	before ultracentrifugation	after ultracentrifugation	before ultracentrifugation	after ultracentrifugation	before ultracentrifugation	after ultracentrifugation
blank niosomes	135.93 ± 0.76	191.23 ± 3.12	80.92 ± 1.61	83.71 ± 0.66	75.20 ± 3.46	71.07 ± 1.88
0.1% OSF3 niosomes	125.90 ± 0.52	200.60 ± 1.15	61.69 ± 1.75	81.32 ± 1.13	80.17 ± 1.72*	72.53 ± 0.45
0.5% OSF3 niosomes	155.70 ± 1.06	192.80 ± 2.69	56.60 ± 1.33	64.71 ± 0.90	80.97 ± 1.07*	71.43 ± 0.76
1.0% OSF3 niosomes	176.53 ± 1.01	522.23 ± 26.21	55.12 ± 1.26	62.71 ± 0.37	78.47 ± 1.76	73.47 ± 1.70
2.0% OSF3 niosomes	222.57 ± 2.44	ND	47.02 ± 2.29	ND	75.10 ± 0.46	71.33 ± 1.56

Note: "ND" was "can not be detected".

1% OSF3 niosomes increased to 522 nm. But, an aggregation of the 2.0% OSF3 niosomes with the non-vesicle morphology was observed under an optical microscopy. The fragile membrane of the 2.0% OSF3 niosomes may be destroyed by the high power of ultracentrifugation. The swollen or the larger size of the blank, 0.1 and 0.5% OSF3 niosomes after centrifugation might be from the rapid dehydration from the vesicles and the rehydration by the reconstitution of the vesicular membrane without size reduction by sonication (Kato et al., 2003).

The morphology of all niosomal formulations before and after the ultracentrifugation was observed by FF-TEM (**Figure 18**). Sizes of niosomes before ultracentrifuged were in the range of 100 - 200 nm with the rough surface. Moreover, the 2.0% OSF3 niosomes had some small vesicles with smooth surface at the size of about 50 nm. The oil droplets suspended outside around the vesicles may affect the white milky appearance of the 2.0% OSF3 niosomes. Both FF-TEM and SAXS analysis indicated the unilamellar niosomal structures of all formulations since no sharp peaks but the broad peaks were observed at the region of q at about 0.8-1.0 nm^{-1}. The scattering profiles of the blank niosomes and niosomes loaded with 0.1, 0.5, 1.0 and 2.0% OSF3 exhibited the similar trends showing that the loaded concentration of OSF3 did not affect the niosomal structures. After centrifugation, all niosomes gave the larger sizes under FF-TEM which complied with the particle size analysis by light scattering.

4.3.3 Zeta potential values

The blank CTAB cationic niosomes showed the zeta potential values at 80.92 mV. The 0.1, 0.5, 1.0 and 2.0% of OSF3 loaded niosomes gave the zeta

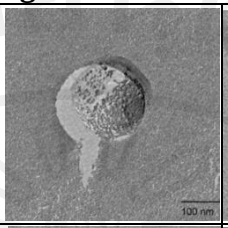
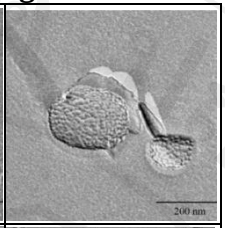
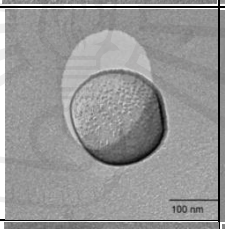
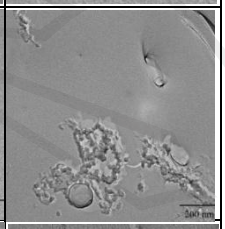
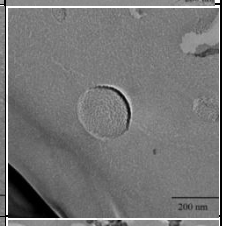
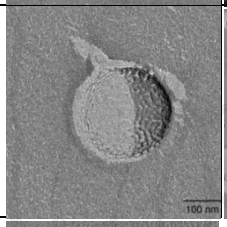
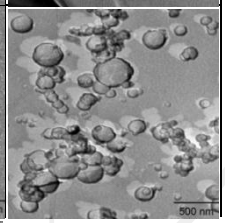
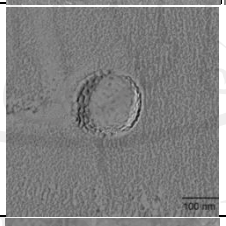
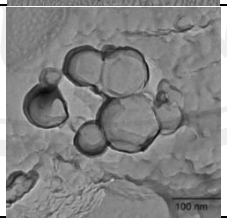
Formulation	Before ultracentrifu-gation	After ultracentrifu-gation
blank niosomes		
0.1% OSF3 niosomes		
0.5% OSF3 niosomes		
1.0% OSF3 niosomes		
2.0% OSF3 niosomes		Not observed
oil droplets in 2.0% OSF3 niosomes		Not observed

Figure 18 FF-TEM images before and after ultracentrifugation of the blank cationic CTAB niosomes and niosomes loaded with 0.1, 0.5, 1.0 and 2.0% (w/v) OSF3. The magnifications were used at 30000× (bar=100 nm), 15000× (bar=200 nm) and 10000× (bar=500nm).

potential values at 61.69, 56.60, 55.12 and 47.02 mV, respectively. The negative charges of the carboxyl group in the unsaturated fatty acids of OSF3 (Leaf et al., 2003) loaded in niosomes may give the more negative zeta potential values to the niosomal membrane. After ultracentrifugation, the blank niosomes indicated similar zeta potential values of 83.71 mV to those of before ultracentrifugation. The zeta potential value of 0.1% OSF3 niosomes was increased from 61.69 mV to 81.32 mV owing to some loaded OSF3 at the membrane which may be centrifuged out. The 0.5 and 1.0% OSF3 niosomes which gave the lower zeta potential values of 64.71 and 62.71 mV, respectively, suggested that some OSF3 may be left at the niosomal membrane that some positive charges of the cationic niosomes were neutralized by the fatty acids in OSF3. However, the 2.0% OSF3 niosomes showed the zeta-potential inside the ± 30 mV range demonstrating the physical unstable dispersion (Gibson et al., 2009). This result agreed with the appearance of this formulation which had the small white flakes precipitated at the bottom. The zeta potential value depends on the movement of the particles (electrophoretic velocities). If the zeta potential is reduced below a certain value (inside ± 30 mV range), the attractive forces between particles due to van der Waals' force, overcome the forces of repulsion and the particles come together to form aggregation (Nutan and Reddy, 2009).

4.3.4 Transition temperatures of the CTAB cationic niosomes

The DSC thermogram analysis of the blank niosomes gave the transitions temperature (Tc) at 75.20 °C **(Table 20).** The Tc of niosomes loaded with 0.1 and 0.5% of OSF3 were significantly increased from the Tc of blank formulation (p <0.05) to 80.17 and 80.97 °C, respectively. The higher Tc might be affected by the Tc of OSF3 (92.19 °C) which both suspended outside the niosomes and loaded in the

niosomal membrane. After the ultracentrifugation, the blank, 0.1, 0.5, 1.0, 2.0% OSF3 niosomes showed the no significant difference of Tc (p <0.05) at 71.07, 72.53, 71.43, 73.47 and 71.33°C, respectively, demonstrating that all niosomal membranes changed from the gel to the liquid crystalline phase at about 70°C. This may indicate that the excess oil outside the niosomes disappeared or had no effect on the Tc of the niosomal membrane.

4.3.5 Microviscosity of the niosomal membrane

The temperature dependence microviscosity trends of all niosomal formulations before and after ultracentrifugation were observed by DSC (**Figure 19A and 19B**). Before ultracentrifugation, the microviscosity of each niosomal formulation gave different fluorescence polarizations. The blank niosomes showed the highest microviscosity indicating the most rigid membrane, followed by the 0.1, 0.5, 1.0 and 2.0% OSF3 niosomes, respectively. The intervention between the unsaturated fatty acids containing in OSF3, Tween 61 and cholesterol at the niosomal surface membrane may lead to the less rigidity of the membrane when the amounts of OSF3 were increased. The packing density of the vesicular membrane is influenced by the large numbers of water molecules around the hydrophilic head groups of Tween 61. When the temperature is increased, these water molecules mobile easily leading to the change in microviscosity of the membrane at high temperatures (Manosroi et al., 2003).

After ultra centrifugation, all niosomal formulations gave similar fluorescence polarization of about 0.3 **(Figure 19B**) indicating the similar membranes rigidity at 25 °C. When the temperature was increased, all systems gave sharp descending lines the

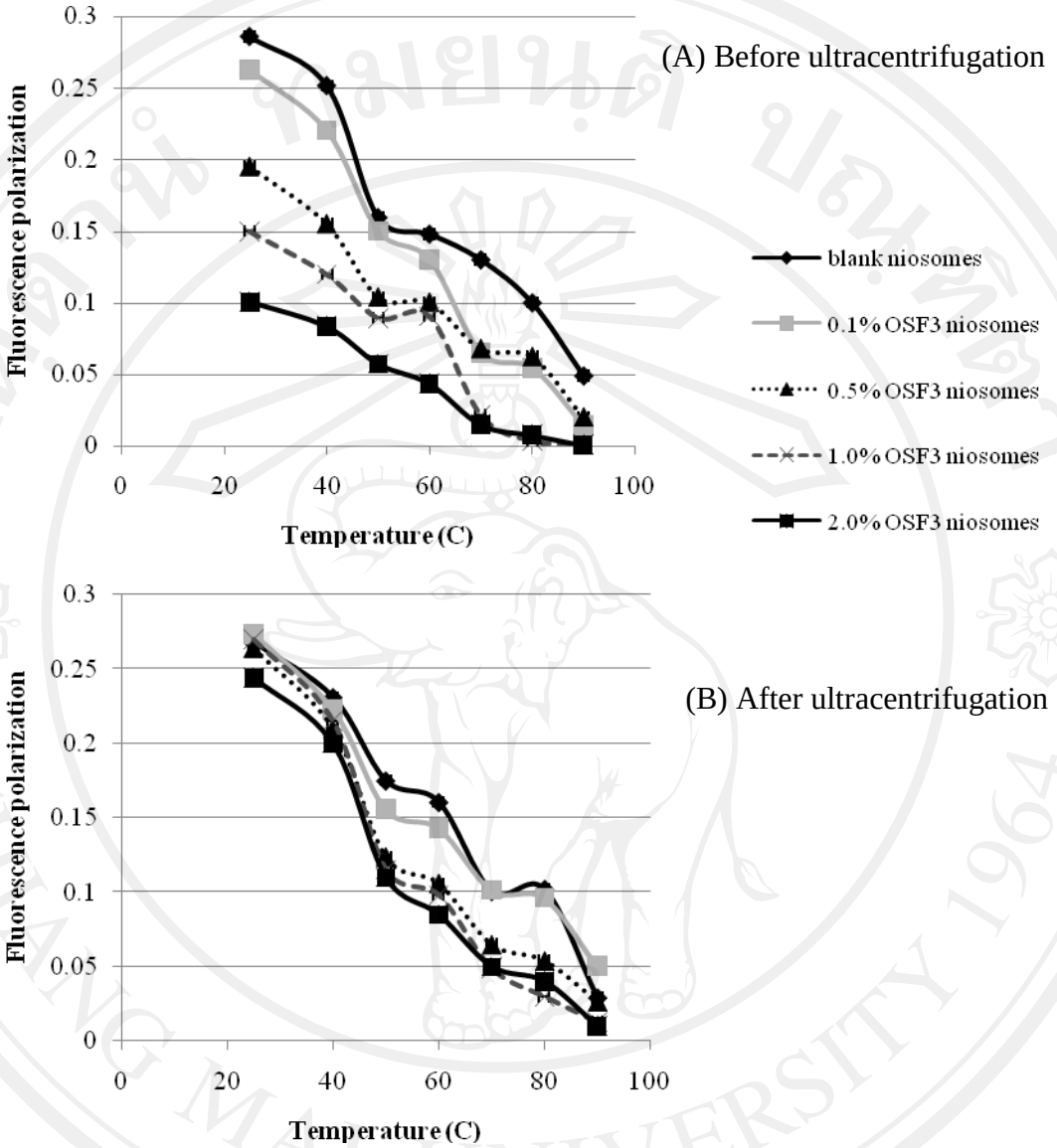


Figure 19 Microviscosities of the blank cationic CTAB niosomes and the niosomes loaded with 0.1, 0.5, 1.0 and 2.0% (w/v) OSF3 before and after ultracentrifugation at 47,000 rpm, 4°C for 90 minutes, (A) before ultracentrifugation, (B) after ultracentrifugation

$$\text{Fluorescence polarization} = \frac{[\text{Fluorescence intensity}_{parallel} - G(\text{Fluorescence intensity}_{vertical})]}{[\text{Fluorescence intensity}_{parallel} + G(\text{Fluorescence intensity}_{vertical})]}$$

same as before ultracentrifugation at about 40 and 70 °C resulting in the rapid change of the membrane rigidity. When the niosomes were stored below 40°C, their membranes were rigid giving the good stability of the loaded OSF3 at the niosomal membrane. Thus, after topical application on the skin with the temperature at about 40°C, the rigidity of the niosomal membrane which slightly changed from the closely packed gel to the loosely packed liquid crystalline structure and resulted in the conformable structure was expected to have better transfollicular penetration property into the hair follicles (Manosroi et al., 2008a).

4.3.6 Micropolarity environment of the niosomal membrane

All niosomal formulations were investigated for micropolarity using the pyrene probe. The results found that the I1/I3 ratios of the blank niosomes and niosomes loaded with 0.1,0.5,1.0 and 2.0% OSF3 gave similar ratios of about 1.00 demonstrating the hydrophobic environment around the vesicles (Kalyanasundaram, 1987) which can be concluded that OSF3 (having hydrophilic property) did not affect the micropolarity environment of the niosomes in comparing to the aqueous solution of pyrene which gave the I1/I3 ratio of 1.80.

From the physico-chemical characteristics of CTAB cationic niosomes loaded with OSF3 at various concentrations, the 0.5% OSF3 niosome was the best formulation because of its high entrapment efficiency of 86.22±1.43%, good physico-chemical characteristic with the vesicular size of about 150 nm, the zeta potential value of about 60 mV and transition temperature of about 40 and 81°C. These suitable properties of the 0.5% OSF3 CTAB cationic niosomes for the development as a transfollicular formulation for anti-hair loss can be anticipated.

Part 5: *In vitro* transfollicular penetration of unsaturated fatty acids of gel OSF3 niosomes in porcine skin by Franz diffusion cells

5.1 Physicochemical stability of gel containing OSF3 niosomes

5.1.1 Physical stability of gel containing niosomes loaded with OSF3

The 2.0% (w/v) OSF3 niosomes was selected to incorporate in gel because of the maximum loading in this formulation which might be advantage for the transfollicular delivery. At this concentration (2.0%, w/v), both loaded and unloaded of OSF3 in the gel OSF3 niosomes formulation without the separation layer could be penetrated through the hair follicles for anti-hair loss. The size of niosomes loaded with OSF3 incorporated in gel (gel OSF3 niosomes) can not be observed since the network structure of the Carbopol gel may interfere with the light scattering intensity resulting in the vesicular size of more than 10 µm. At 25°C, the zeta potential of the gel OSF3 niosomes were within the stable dispersion range of -40.33, -39.79, -37.03 and -36.28 mV at initial, 1, 2 and 3 months respectively. The negative charges of the carboxyl groups in the Carbopol polymer structure may neutralize the positive charges of the cationic niosomes (48.00 mV) resulting in the less negative value of the gel OSF3 niosomes (-40.33 mV) than the gel base (-55.51 mV). As known, Carbopol is a polymer of the acrylic acid cross-linked with the polyalkenyl ethers or divinyl glycol providing carboxyl groups by the acrylic acid backbone (Avinash et al., 2006).

5.1.2 Chemical stability of unsaturated fatty acids in gel containing OSF3 loaded in niosomes

The percentages (%) remaining of the unsaturated fatty acid contents (gamma-linolenic acid, linoleic acid and oleic acid) in gel OSF3 niosomes were

presented in **Figure 17**. The gamma-linolenic acid contents (73.34, 77.68, 70.43% w/w) were the least followed by linoleic acid (82.56, 85.34, 75.67% w/w) and oleic acid contents (85.21, 89.47, 77.89% w/w), respectively, when stored at 4, 25, 45°C for 3 months. This trend was similar to the systems of OSF3 solution and OSF3 niosomes. Oleic acid which gave the highest contents in the gel OSF3 niosomes at all storage temperatures may be owing to its least numbers of double bonds (18:1, *n*-9) with more chemical stability than gamma-linolenic acid and linoleic acid with more numbers of double bonds.

At 4 and 25°C after stored for 3 months, the gel OSF3 niosomes gave the similar content of the total unsaturated fatty acids of more than 80%. However, at 45 °C, this system showed lower total unsaturated fatty acids content at about 74%. The high storage temperature may accelerate the oxidation reaction of the double bonds. The gel OSF3 niosomes contained higher content of the total unsaturated fatty acids (80.37, 84.16 and 74.66%) than the OSF3 niosomes (80.56, 80.26 and 73.82%) after stored at 4, 25 and 45 °C for 3 months, respectively. The unsaturated fatty acids in the gel OSF3 niosomes appeared to be the most stable ones followed by those in the OSF3 niosomes and the OSF3 solution, respectively. The gel structure and the niosomes may have the synergistic effects to obstruct the exposure of the unsaturated fatty acids to high temperature (Perez-Marcos et al., 1994).

5.2 *In vitro* transfollicular penetration of unsaturated fatty acids of gel OSF3 niosomes in porcine skin

The cumulative amounts of gamma-linolenic acid, linoleic acid and oleic acid as well as the total unsaturated fatty acids (ng/cm^2) in skin and the receiver chamber at 0, 1, 2, 4 and 6 hours were shown in **Figure 20**. The cumulative amounts (ng/cm^2),

fluxes (ng/cm^2/hour) and follicular penetration per one hair follicle (ng/one hair follicles) by follicular closing technique using Franz diffusion cells at 6 hours of unsaturated fatty acids in various formulations containing OSF3 were presented in **Table 21.** The cumulative amounts (ng/cm^2, n=3) through skin of the unsaturated fatty acids in all formulations were increased with times. In the skin after 6 hours, the OSF3 niosomes (120.27 ng/cm^2) and gel OSF3 niosomes (118.17 ng/cm^2) exhibited higher cumulative amounts of the total unsaturated fatty acids than those from the gel OSF3 (15.34 ng/cm^2) and OSF3 solution (20.31 ng/cm^2) of about 8 and 6 times, respectively. This indicated that niosomes may facilitate the transfollicular delivery of the unsaturated fatty acids. Interestingly, even the OSF3 contained the lowest amount of gamma-linolenic acid (7.52%, w/w) in comparing to oleic acid (42.17%, w/w) and linoleic acid (49.25%, w/w), gamma-linolenic acid (65.85 ng/cm^2) which is a 18:3 (n−6) unsaturated fatty acid in the OSF3 niosomes penetrated through the hair follicle better than linoleic acid (18:2, (n-6), 31.86 ng/cm^2) and oleic acid (18:1, (n-9), 22.55 ng/cm^2) of about 2.1 and 2.9 times, respectively. This has suggested that the amounts of the gamma-linolenic acid in OSF3 did not affect the penetration ability. But, the smaller flexible molecular structure of gamma-linolenic acid with 3 double bonds which can fold into a more compact molecule than linoleic acid (2 double bonds) and oleic acid (1 double bond) gave the better penetration through hair follicles. Thus, the high penetration through hair follicle of the unsaturated fatty acids may not be by the concentration, but by the smaller flexible molecule. However, the cumulative amounts of linoleic acid (35.18 ng/cm^2) and oleic acid (33.62 ng/cm^2) in gel OSF3 niosomes were more than those in OSF3 niosomes (31.86 and 22.55 ng/cm^2). The occlusion effect of the gel structure may retard the penetration of the

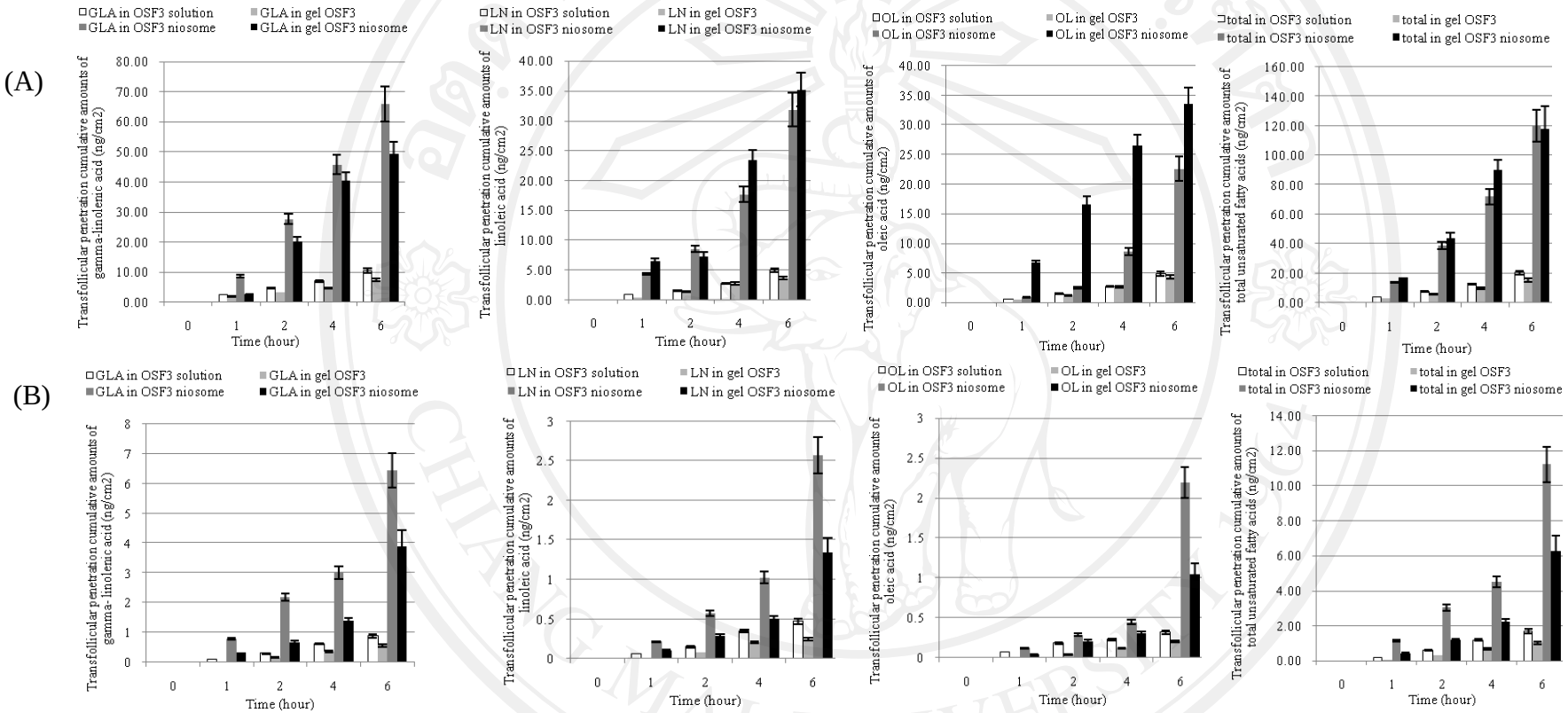


Figure 20 Cumulative amounts of gamma-linolenic acid, linoleic acid, oleic acid and the total unsaturated fatty acids (ng/cm^2) in skin (A) and the receiver (B) by follicular closing technique using Franz diffusion cells at 0, 1, 2, 4 and 6 hours

Table 21 The cumulative amounts (ng/cm^2), fluxes (ng/cm^2/hour) and follicular penetration per one hair follicle (ng/one hair follicle)] by follicular closing technique using Franz diffusion cells at 6 hours of gamma-linolenic acid (GLA), linoleic acid (LN) and oleic acid (OL) from various formulations containing *Oryza sativa* semi purified fraction 3 (OSF3)

formulation		skin			receiver		
		cumulative amounts (ng/cm2)	fluxes (ng/cm2/hour)	follicular penetration per one hair follicle (ng/one hair follicle)	cumulative amounts (ng/cm2)	fluxes (ng/cm2/hour)	follicular penetration per one hair follicle (ng/one hair follicle)
OSF3 solution	GLA	10.480	1.747	0.161	0.890	0.148	0.014
	LN	4.970	0.828	0.076	0.470	0.078	0.007
	OL	4.860	0.810	0.075	0.320	0.053	0.005
	total	20.310	3.385	0.312	1.680	0.280	0.026
gel OSF3	GLA	7.352	1.225	0.113	0.569	0.095	0.009
	LN	3.630	0.605	0.056	0.244	0.041	0.004
	OL	4.360	0.727	0.067	0.203	0.034	0.003
	total	15.342	2.557	0.236	1.016	0.169	0.016
OSF3 niosomes	GLA	65.854	10.976	1.013	6.434	1.072	0.099
	LN	31.860	5.310	0.490	2.566	0.428	0.039
	OL	22.554	3.759	0.347	2.195	0.366	0.034
	total	120.268	20.045	1.849	11.196	1.866	0.172
gel OSF3 niosomes	GLA	49.370	8.228	0.759	3.890	0.648	0.060
	LN	35.180	5.863	0.541	1.340	0.223	0.021
	OL	33.620	5.603	0.517	1.040	0.173	0.016
	total	118.170	19.695	1.817	6.270	1.045	0.096

Note: Each value represented mean of three experiments.

OSF3 solution= *Oryza sativa* semi purified fraction 3 dispersed in 70% propylene glycol; gel OSF3= gel containing non-loaded 2.00% w/v of OSF3; OSF3 niosomes = niosomes loaded with 2.00% w/v of OSF3; Gel OSF3 niosomes = gel containing niosomes loaded with 2.00% w/v of OSF3.

more compact fatty acid (gamma-linolenic acid), but enhanced the penetration through the hair follicles of the less compact molecules having less double bonds (linoleic and oleic acid). The OSF3 niosomes (11.20 ng/cm^2) exhibited more cumulative amount of the total unsaturated fatty acids in the receiving solution than those in the gel OSF3 niosomes (6.27 ng/cm^2), OSF3 solution (1.68 ng/cm^2) and gel OSF3 (1.02 ng/cm^2) of about 2, 7 and 11 times, respectively. For all formulations, gamma-linolenic acid appeared to penetrate into the receiver more than linoleic acid and oleic acid of about 3 times resulting in higher transfollicular penetration per one hair follicle and fluxes through hair follicles. However, different penetration patterns of linoleic acid and oleic acid were observed in the receiver. For the OSF3 niosomes, the unsaturated fatty acids including gamma-linolenic acid, linoleic acid and oleic acid showed the highest cumulative amounts in the receiver of about 6.43, 2.57 and 2.20 ng/cm^2, respectively. Moreover, the cumulative amounts in the skin of the total unsaturated fatty acids in OSF3 niosomes, gel OSF3 niosomes, gel OSF3 and OSF3 solution were higher than those in the receiver compartment of about 11, 18, 15 and 12 times, respectively. This might be due to not only the absorption enhancement effects from niosomes (Tabbakhian et al., 2006), but also the more contact area to the skin of the niosomes itself than the niosomes incorporated in gel (gel OSF3 niosomes). Also, the diffusion through the skin of the fatty acids may be restricted by the polymeric network of the gel (Glavas-Dodov et al., 2003) thereby saturating the unsaturated fatty acids within the skin. In fact, the release of the drugs or bioactives from the extract out of the vesicles incorporated in gel formulations has been reported to be much slower than those from the vesicle itself (Ning et al., 2005). Moreover, the gel OSF3 niosomes (6.27 ng/cm^2) exhibited 2 times lower cumulative amount of

the total unsaturated fatty acids than the OSF3 niosomes (11.20 ng/cm^2) indicating of less systemic risk.

This study has suggested that the gel incorporated with OSF3 loaded in niosomes (gel OSF3 niosomes) appeared to be the most suitable system for OSF3 to be used in androgenic alopecia treatment (Ruksiriwanich et al., 2011). Although this system gave lower transfollicular penetration of unsaturated fatty acid into the receiver compartment than the OSF3 niosomes, it had the proper viscosity for convenient topical use and superior occlusion effect that will be beneficial for the saturation in the skin with less risk of systemic effects.

Part 6: *In vivo* hair growth promotion activity of gel containing cationic niosomes loaded with OSF3

6.1 Toxicity evaluation of the formulations containing OSF3

OSF3 which exhibited high contents of unsaturated fatty acids with high antioxidative and the highest *in vitro* 5α-reductase inhibition activities was selected to load in cationic CTAB niosomes and incorporated in gel. Gel containing OSF3 niosomes (gel OSF3 niosomes) which indicated high transfollicular penetration in porcine skin, but not in the receiving compartment with the advantage of low systemic risk was investigated for *in vitro* and *in vivo* toxicity in comparing to OSF3 niosomes, OSF3 solution and the positive control (dutasteride). Cytotoxicity of OSF3 in various formulations on viability of human skin fibroblasts (passage 14th) at 24 h incubation was presented in **Table 22**. Gel OSF3 niosomes gave lower toxicity than OSF3 niosomes. Gel base showed the lowest toxicity with the % cell viability of 95.48%. The toxicity of the gel was slightly increased when incorporated with OSF3 (gel OSF3) with the % cell viability of 93.18%, but not significant. This may be due to

Table 22 ***In vitro*** **cytotoxicity on human skin fibroblasts (passage 14th) and** ***in vivo*** **primary irritation index (PII) and category of irritation on rabbit skin based on PII of various formulations containing OSF3.**

sample	*in vitro*	*in vivo*			
	% cell viability	Primary Irritation Index (PII)			category of irritation based on PII
		24 h	48 h	72 h	
gel OSF3 niosomes	92.14±2.34	0.00	0.00	0.00	Negligible
gel OSF3	93.18±3.17	0.00	0.00	0.00	Negligible
gel base	95.48±2.12	0.00	0.00	0.00	Negligible
blank niosomes	85.23± 1.74	0.00	0.00	0.00	Negligible
OSF3 niosomes	80.19±3.28	0.33	0.00	0.00	Negligible
OSF3 solution	89.65±2.89	0.00	0.00	0.00	Negligible
5% sodium lauryl sulfate (positive control)	NA	1.22	1.00	0.78	Slight irritation
untreated area (negative control)	NA	0.00	0.00	0.00	Negligible
vitamin C	97.62±4.41	NA	NA	NA	NA

Note: "NA" means "not applicable"

The grading scale for irritation on rabbit's skin following OECD Test Guideline 404

Primary irritation index (PII) = [(Σ erythema grade at 24/48/72 h + Σ edema grade at 24/48/72 h)/ 3 × number of animals].

some toxicity of OSF3. The blank cationic niosomes (85.23% cell viabilty) gave lower toxicity than OSF3 niosomes (80.19% cell viabilty). The toxicity of OSF3 niosomes may be from both OSF3 and the cationic lipid CTAB which has been used as an antiseptic and disinfectant (Nicoletti *et al.*, 1993). The cell viability of OSF3 niosomes was still more than 80% (Hegde *et al.*, 2006). However, when incorporated in gel formulation (gel OSF3 niosomes), the toxic of OSF3 niosomes was reduced (92.14% cell viability). For the *in vivo* rabbit skin irritation by the closed patch test, the calculated PIIs of all formulations at 72 h were in the range of 0.00–0.33 while that of the positive control (5% SLS) was in the range of 0.78–1.22 demonstrating of slight irritant **(Table 22).** OSF3 niosomes gave slight *in vitro* cytotoxicity and negligible of irritation in rabbit skin. But, when incorporated in gel, gel OSF3 niosomes appeared to be suitable topical formulation because of the *in vitro* non-toxicity and *in vivo* non-irritation. The gel structure may protect the rabbit skin from directly contacted with the OSF3 niosomes. When incorporated in gel, the skin irritation from OSF3 niosomes appeared to be retarded.

6.2 Evaluation of the *in vivo* hair growth promotion activity of OSF3

6.2.1 Observation and photography

Figure 21 indicated the hair growth promotion activity on C57BL/6 mice of OSF3 containing in various formulations after application for 21 days and 21 days of the wash-out period. Hair growth score was evaluated every week as shown in **Figure 22A**. The hair of the mice treated with the OSF3 niosomes gave the highest hair growth score of 3.5 at day 21st which were higher than gel OSF3 niosomes (3.0), OSF3 solution (3.0), the positive control dutasteride (2.0) and the negative control (no treatment) (0.8) of 1.16, 1.16, 1.75 and 4.38 times, respectively. After the wash-out

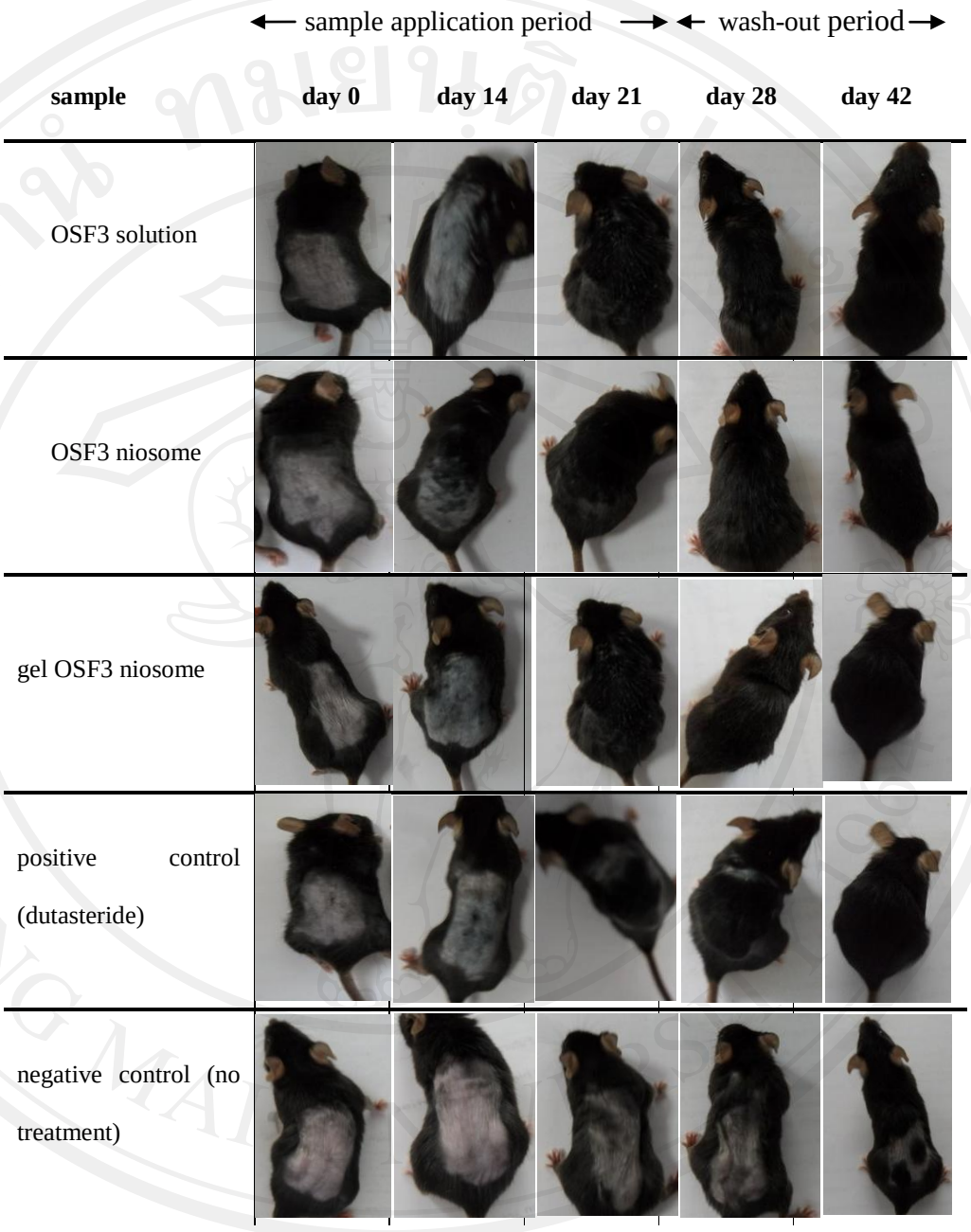


Figure 21 Hair growth promotion activity on C57BL/6 mice of OSF3 (*Oryza sativa* fraction No.3) containing in various formulations [OSF3 solution (OSF3 dissolve in 95%, v/v ethanol), OSF3 niosomes (OSF3 loaded in CTAB niosomes) and gel OSF3 niosomes (gel containing OSF3 loaded in CTAB niosomes)] after daily topical application for 21 days. Photographs of the dorsal skin of the mice were taken every week. Hair density and hair recovery were compared optically between the treated groups, positive control (dutasteride solution) and negative control (no treatment).

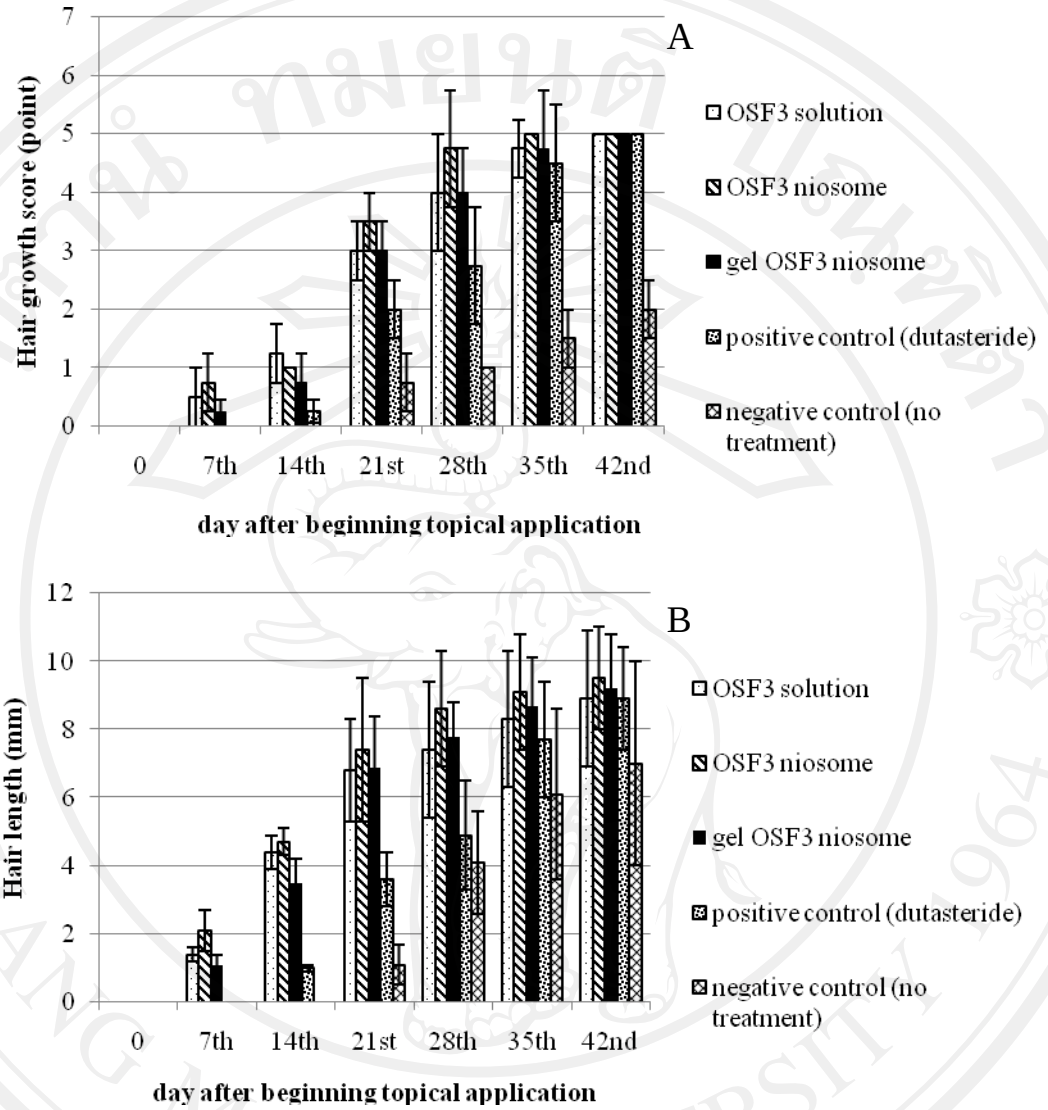


Figure 22 Evaluation of hair growth promotion activity (hair growth score and hair length) in C57BL/6 mice of OSF3 containing in various formulations. (A): Hair growth scores from optical observation during the experiment evaluated from the following score criteria: score 0 (no hair growth), score 1 (less than 20% growth) , score 2 (20% to less than 40% growth), score 3 (40% to less than 60% growth), score 4 (60% to less than 80% growth) and score 5 (80% to 100% growth). (B): Hair length was measured from the hair randomly plucked from the mice dorsal on day 7^{th}, 14^{th}, 21^{st}, 28^{th}, 35^{th} and 42^{nd} of sample application and wash-out period. The average (mean±S.D.) hair length was determined from 10 hair shafts of each mice (n=4) in each group.

period at day 42nd, all OSF3 formulations (OSF3 solution, OSF3 niosomes and gel OSF3 niosomes) and the positive control group (dutasteride) gave the hair growth score of 5.0, while the negative control (no treatment) indicated only 2.0. Moreover, OSF3 niosomes had the recovery of the hair with the shortest time of 34.75±1.26 days while gel OSF3 niosomes, OSF3 solution and dutasteride were at 37.50±1.29, 38.50±0.58 and 41.00±0.82 days after sample application, respectively.

As reported, the dorsal hair of the C57BL/6 mouse was known to have a time-synchronized hair growth cycle. Depilated mice skin in telogen phase was pink and will be darkens along with the anagen initiation and then turn into gray (Chase, 1954; Paus et al., 1990). From weeks 7 to 12 of the postnatal life, these mice were in the telogen or resting stage of the hair cycle which can provide a large window of time to assay for anagen induction in the absence of endogenous hair growth (Paladini et al., 2005). In this study, the pink dorsal skin of mice treated with OSF3 solution, OSF3 niosomes, gel OSF3 niosomes and dutasteride turned to light gray after 7 days and dark gray after 14 days of sample application indicating that the hair follicles turned into the anagen phase. However, the negative control group (no treatment, gel base, 95% ethanol and distilled water) were still in pink color (telogen phase). This may conclude that OSF3 in various formulations can induce the hair follicle from the telogen to the anagen phase. The visible hair was observed and the dorsal skin was in dark gray on day 14th after sample application in the group treated with OSF3 niosomes. More hair shafts were observed in all OSF3 groups (OSF3 solution, OSF3 niosomes and gel OSF3 niosomes) than the dutasteride group on day 21st after sample application. Meanwhile, the hair of the negative control groups (no treatment, gel base, 95% ethanol and distilled water group) turned from pink to gray color and the

hair shaft was visible after day 21 of sample application. This indicated that OSF3 may induce hair growth through the conversion of the early telogen to anagen hair follicles in C57BL/6 mice. Moreover, the lower activity of dutasteride than gel containing OSF3 loaded in niosomes might be from the low skin penetration of dutasteride. The high molecular weight (MW=528.5), solubility in ethanol (44 mg/ml) but insolubility in water of dutasteride when in solution but not in niosomal formulation may affect the difficulty of dutasteride to penetrate through the skin.

6.2.2 Hair length determination

Hair length of C57BL/6 mice in each group was shown in **Figure 22B**. OSF3 niosomes gave the longest hair length after 21 and 42 days of sample application at 7.4 and 9.5 mm, respectively. After 21 days of sample application, OSF3 niosomes (7.4 mm) indicated longer hair length than the gel OSF3 niosomes (6.9 mm), OSF3 solution (6.8 mm), dutasteride (3.6 mm) and negative control (1.7 mm) of 1.07, 1.09, 2.06 and 4.35 times, respectively. This might be due to the slight irritation on rabbit skin of OSF3 niosomes with the PII after 24 hours of 0.33 which may cause some redness and counter irritation that can synergist the hair growth promotion of OSF3. As known, OSF3 can inhibit 5α-reductase enzyme, a key enzyme to produce DHT, which caused hair loss (Ruksiriwanich et al., 2011). Gel incorporated with OSF3 loaded in niosomes (gel OSF3 niosomes) indicated the best hair growth promotion activity with no irritation and cytotoxicity and also exhibited an efficient hair growth promotion activity in C57BL/6 mice with the hair growth score of 3.0 and the mean hair length of 6.9 mm at day 21 after sample application, which were not significant different from the OSF3 niosomes. After the wash-out

period at day 42 after application, all formulations also gave longer hair than at the initial (after shaved) with the hair length of more than 7 mm.

6.2.3 Histological studies

Histological analysis of the skin specimens of C57BL/6 mice at day 21st after sample application was shown in **Figure 23**. For all OSF3 formulations, OSF3 niosomes exhibited the highest numbers of hair follicles in the dermis layer in comparing to the negative control group **(Figure 23B)**. All samples in the negative control group (no treatment, distilled water, 95% ethanol and gel base) gave similar

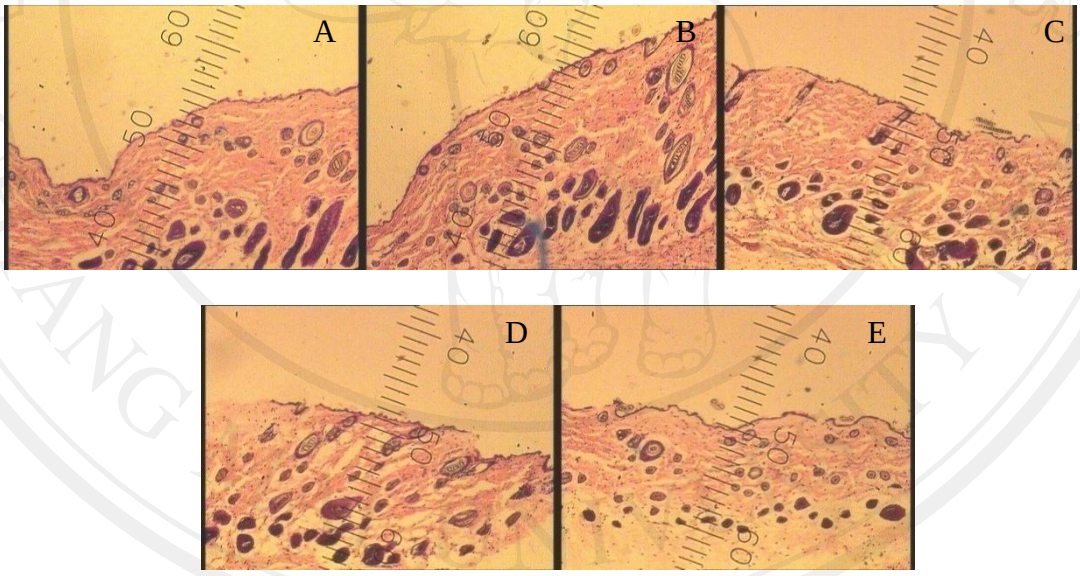


Figure 23 Histological analysis of the dorsal skin specimens of C57BL/6 mice at day 21 treated with various formulations containing OSF3, A: OSF3 solution, B: OSF3 niosomes, C: gel OSF3 niosomes, D: positive control (dutasteride) and E: negative control (no treatment).

histological skin patterns with the early anagen phase at day 21st after sample application. This may be due to the anagen induction from depilation (Muller-Rover

et al., 2001), but not from the base compositions. The skin treated with gel OSF3 niosomes and OSF3 solution indicated slightly lower amount of the active hair follicles in the dermis layer than those treated with the OSF3 niosomes, but not significant. The higher amount hair shafts and hair follicles in anagen phase were significantly observed in the skin specimens treated with all OSF3 formulations and dutasteride than those of the negative control group. The treatment of all OSF3 formulations appeared to produce the normal hair follicles containing well-differentiated straight hair shafts that usually emerged from hair follicles reaching the skin's surface. From the histological analysis, it was suggested that gel OSF3 niosomes had the anagen induction effect and gave higher amount of hair follicles in the dermis layer that can generate the well-differentiated straight hair shafts from hair follicles. Thus, this study has suggested that the best topical formulation for anti-hair loss and hair growth promotion appeared to be the gel OSF3 niosomes.