CHAPTER 4

CONCLUSION

The results from this study can be concluded as the followings:

Part 1: Crude extracts preparation and their biological activities

1.1 The optimized condition for the $scCO_2$ extraction was 200 bar, 40°C and 25% w/v of 95% v/v ethanol which was used to extract the selected 10 plants. The rice bran crude extract prepared by $scCO_2$ (with 25% w/v of 95% v/v ethanol) gave lower yields (11.21 ± 0.54 % w/w), but higher unsaturated fatty acid contents (gamma-linolenic acid, linoleic acid and oleic acid at 5.67 ± 0.52, 23.62 ± 2.62 and 27.28 ± 1.22 % w/w, respectively) and more total phenolic contents in the form of gallic acid (0.65 ± 0.05 mg/g) than that from the ethanolic maceration. Moreover, the rice bran crude extracts from $scCO_2$ and ethanolic maceration showed no significant difference of antioxidative, tyrosinase inhibition and cell proliferation activity on aged normal human skin fibroblasts. The selected 10 plants including *A. hypogaea* (peanut), *C. tinctorius* (safflower), *G. max* . (soybean), *H. annuus* (sunflower), *L. usitatissimum* (flax), *N. nucifera* (lotus), *O. sativa* (rice), *S. indicum* (sesame), *S. bicolor* (sorghum) and *Z. may* (corn) were extracted by $scCO_2$ and ethanolic maceration methods and compared the extraction yields and biological activities.

1.2 The crude extracts from the three edible plants, including *O. sativa*, *C. tinctorius* and *S. bicolor* prepared by $scCO_2$ method which showed high unsaturated

fatty acids contents and anti-oxidative activities, including the DPPH radical scavenging, lipid peroxidation inhibition, metal ion chelating, tyrosinase inhibition activities, stimulation index on human normal skin fibroblast, were selected from the ten edible plants to prepare the semi-purified fractions.

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

2.1 The *O. sativa, C. tinctorius* and *S. bicolor* crude extracts were semi-purified by column chromatography that eluted with petroleum ether/ethyl acetate (8:1) at the flow rate of 1 ml/min. Each crude extract composed of 4 semi-purified fractions.

2.2 Fraction No.3 of *O. sativa* crude extract (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

2.3 OSF3, the *O. sativa* and *C. tinctorius* crude extracts demonstrated high antioxidant activities (A_F, A_L and A_C) and gave high total phenolic contents which showed positive linear correlations to the 5α-reductase inhibition activity (5AR) with $r>0.95$ ($p<0.05$) that can reinforce the healthy tissue around the hair follicles leading to the reduction of the shedding hair before the telogen phase.

2.4 OSF3 indicated 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), high anti-oxidative activities with high stimulation index on human normal skin fibroblast (stimulation index= 1.01 ± 0.34) and the highest 5α-reductase (type 1) inhibition (93.33 ± 10.93 % of control) on DU-145 prostate cancer cell line.

2.5 The linoleic acid content (LN) and the total unsaturated fatty acid content (TUC) in OSF3 were significantly related to the 5α-reductase inhibition activity (5AR) (LN and 5AR; $r = 1.00$, $p<0.01$) and (TUC and 5AR; $r = 1.00$, $p<0.01$).

2.6 The positive correlation between 5α-reductase inhibition and antioxidant activity of OSF3, 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; $r = 0.89$, $p<0.05$) were observed.

2.7 The genetic anti- hair loss activity of the crude extracts and fractions prepared by $scCO_2$ from these three plants, especially OSF3 can be anticipated since they inhibited the 5α-reductase mRNA production which can lead to the formation of 5α-reductase type 1 protein, the major cause of hair loss.

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

3.1 The blank neutral niosomes was prepared from Tween 61/cholesterol (1:1 molar ratio) by chloroform film method and $scCO_2$. The maximum loading of the *O. sativa* crude extracts in neutral niosomes prepared by both methods was 0.25 % w/w.

3.2 The physical characteristics including the vesicular size, morphology, phase transition temperature and the microviscosity of the niosomes loaded and not loaded with the *O. sativa* crude extracts prepared by $scCO_2$ technique were not significant different from those by the chloroform film method. The morphology of all niosomes were in unilamellar structure investigated by TEM with the average

particle size of 60.34 ± 30.91 nm. For all niosomes, the phase transition (gel-liquid) temperature at about 80°C and the gradual decreasing trend of the vesicular membrane microviscosity with increased temperature were observed.

3.3 The supercritical carbon dioxide fluid ($scCO_2$) was selected for the further preparation of niosomes loaded with the extract because of not only its environmental friendly, but also its less step requirement as well.

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

4.1 The blank cationic niosomes with the composition at 20 mM of Tween61 mixed with cholesterol and cationic surfactants (CTAB, CPC, SA, BZKC, BZT and DDAB) at 1:1:0.05, 1:1:0.25 and 1:1:0.5 molar ratios were prepared. The best physical stability (size and zeta potential) and low toxicity on human skin fibroblast of the three blank niosomes including cationic blank niosomes [Tween61/ cholesterol/ cationic surfactants (CTAB, BZKC) at 1:1:0.5 molar ratios] and the neutral blank niosomes (Tween61/cholesterol at 1:1 molar ratio) were selected to load with the OSF3.

4.2 CTAB niosomes at 1:1:0.5 molar ratio loaded with OSF3 were white dispersion with no layer separation or color change and gave the particle size of 156.07, 164.40, 174.59 nm and the zeta potential values of 54.37, 56.70. 55.43 mV when stored at 45°C for 1, 2 and 3 months, respectively.

4.3 OSF3 was loaded in CTAB cationic niosomes (Tween61/ cholesterol/CTAB) at 0.1, 0.5, 1.0 and 2.0% (w/v). The entrapment efficiency of 0.5 % of OSF3 niosomes was 86.22±1.43%. The white milky translucent appearance was

found in 2.0% OSF3 niosomes. The niosomal sizes were slightly increased from 120 to 220 nm after loaded with various concentrations of OSF3. OSF3 may deposit at the niosomal membrane. The zeta potential value of blank niosomes was decreased from 80 mV to the range of 40-60 mV after loaded with OSF3. Both FF-TEM and SAXS analysis of all niosomal formulations showed the unilamellar niosomes. The transitions temperature (Tc) of the niosomes significant increased from 75 to 80 °C when loaded with 0.1 and 0.5% OSF3. Moreover, the blank niosomes gave the highest microviscosity indicating the most rigid membrane at 25 °C and followed by 0.1, 0.5, 1.0 and 2.0% OSF3 niosomes, respectively. The fluorescence polarizations of all niosomal formulations exhibited the sharp descending phases at about 40 and 70 °C.

4.4 After ultra-centrifugation to eliminate the non-loaded negatively charged OSF3, sizes of the blank, 0.1 and 0.5% OSF3 niosomes were increased from 120 to 200 nm, while 1% OSF3 niosomes increased from 170 to 522 nm. The zeta potential values of 0.1, 0.5 and 1% OSF3 niosomes were increased. All niosomal formulations showed the same transition temperatures at about 71 °C and the same microviscosities at 25 °C.

4.5 Hence, the 0.5% OSF3 niosome was the best formulation which can be further developed as an anti-hair loss product because of its high physico-chemical characteristics.

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

5.1 The gel incorporated with OSF3 loaded in CTAB cationic niosomes (gel OSF3 niosomes) was developed by using 2.0% (w/v) OSF3 niosomes since it is the maximum loading of OSF3 in niosomes.

5.2 The gel OSF3 niosomes, OSF3 niosomes, gel OSF3 and OSF3 solution were investigated for physicochemical characteristics. Gel OSF3 niosomes demonstrated physicochemical stability with the zeta potential values of -36.28 mV and the total unsaturated fatty acid contents of more than 84% after stored at 25 °C for 3 months.

5.3 For transfollicular penetration through porcine skin using follicular closing technique by Franz diffusion cells, although gel OSF3 niosomes gave less transfollicular penetration of the unsaturated fatty acids into the receiver compartment than the OSF3 niosome, it was a more convenient system for topical use because of the synergistic transfollicular enhancement of the unsaturated fatty acids (the key bioactive compounds for 5α-reductase inhibition) by the niosomes and the polymer structure of the gel formulation as well as the less risk of the systemic effect than the OSF3 niosomes.

5.4 Thus, OSF3 loaded in CTAB cationic niosomes and incorporated in gel (gel OSF3 niosomes) which can be developed as an anti-androgenic alopecia product was selected for the further *in vivo* hair growth promotion investigation.

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

6.1 Various formulations containing 2.0% (w/v) of OSF3 (OSF3 solution, OSF3 niosomes, gel OSF3 niosomes) were investigated for *in vitro* cytotoxicity on human skin fibroblasts and *in vivo* irritation test on rabbit skin. Gel OSF3 niosomes exhibited lower toxicity both *in vitro* and *in vivo* than OSF3 niosomes owing to the gel structure that may protect the skin from the toxicity of the cationic niosomes.

6.2 For the *in vivo* hair growth promoting activity in C57BL/6 mice model, gel OSF3 niosomes exhibited similar anagen induction to OSF3 niosomes in C57BL/6 mice. Moreover, gel OSF3 niosomes exhibited higher *in vivo* hair growth promotion activity with the hair growth score of 3.0 and the mean hair length of 6.9 mm at day 21st of sample application than the standard dutasteride of 1.50 and 1.92 times, respectively. From the histological analysis, all formulations containing OSF3 including gel OSF3 niosomes induced the hair follicle to differentiate from telogen to anagen phase resulting in the change of the pink to the gray skin and the generation of the normal hair follicles containing well-differentiated straight hair shafts.

6.3 The hair growth promotion of gel containing 2.0% w/v OSF3 loaded in cationic niosomes was the best formulation for OSF3 to develop as a suitable topical product for hair growth promotion because of its non-toxicity, high hair growth score, long hair length and producing anagen induction in hair follicle.

In summary, the *O. sativa* bran extract which was semi-purified to be OSF3 was the best natural source for the anti-hair loss treatment because of its highest 5α-reductase (type 1) inhibition activity. The OSF3 was loaded in the cationic CTAB

niosome since it showed the stable appearances throughout 3 months after stored at 4, 25 and 45°C to develop as a transfollicular delivery product. The *in vitro* and *in vivo* toxicity of the OSF3 niosome was reduced by its incorporation in the gel formulation (gel OSF3 niosomes). Gel OSF3 niosomes exhibited the high *in vivo* hair growth promotion with the induction of the hair follicle to differentiate from telogen to anagen in C57BL/6 mice. Furthermore, gel OSF3 niosomes are expected to be used as both for prevention and treatment of anti-hair loss in the early stage of androgenic alopecia patients whom still have the lived hair follicles.

For further research suggestions, the gel OSF3 niosomes should be further investigated for hair growth promotion activity in the volunteers especially those with hair loss problems. Also, the preparation of OSF3 by this study may be difficult to scaling up for commercial production. Besides the 5α-reductase inhibition activity, gamma-oryzanol which has antioxidant activity might have the synergistic effect of hair growth promotion by reinforcing the healthy tissue around the hair follicles. So, the gamma-oryzanol contents in the OSF3 should be determined and the standard gamma-oryzanol should be used as a positive control in the *in vivo* hair growth promotion test. The lower *in vivo* hair growth promotion activity of dutasteride in this study might be from the low skin absorption of dutasteride solution due to its high molecular weight and low hydrophilic property. The better penetration formulation of dutasteride should be developed to compare the *in vivo* hair growth promotion activity. In addition, the purity of the unsaturated fatty acids used in this study should be investigated since it might be related to the hair growth promotion activities. As reported previously, the higher purity of unsaturated fatty acids (more than 99%) gave the higher improvement of atopic dermatitis (Aburai et al., 2011). Recently, the N-

myristoyl transferase enzyme was reported to be related to aging and also the hair loss process. The N-myristoyl transferase inhibition activity of OSF3 might be thus valuable for further investigation for its anti-hair loss mechanism.