CHAPTER 4

CONCLUSION

This study can be summarized as the following:

1. Four methyl esters of saturated fatty acids, including lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0), were synthesized by Fischer esterification using acid as a catalyst. The obtained methyl esters were purified by column chromatography and then identified by Fourier transform infrared spectroscopy (FTIR) and gas chromatography-mass spectrometry (GC/MS). Both FTIR and GC/MS spectra indicated that all synthesized compounds were methyl ester derivatives of their corresponding saturated fatty acids.

2. The cytotoxicity as well as the melanin induction activity of four methyl esters, including lauric acid methyl ester (LM), myristic acid methyl ester (MM), palmitic acid methyl ester (PM) and stearic acid methyl ester (SM) were compared to their parent saturated fatty acids in B16F10 melanoma cells. Methyl esters demonstrated higher cell viability than their parent saturated fatty acids, owing to the less acidity of esters than saturated fatty acids. Among 12-18 carbons composing of esters and saturated fatty acids, 12-14 carbons both in the esters and saturated fatty acids showed less cytotoxicity than the compounds with higher carbons. This may be resulted from higher lipophilicity and lipid solubility of long carbon chains than the short carbon chains. Also, the long carbon chain may penetrate through the cell membrane which is consisted of the lipid soluble constituents, such as phospholipids, ceramides and cholesterol, more efficient than the short carbon chain, thereby reducing cell viability.

3. For the melanogenesis induction assay, MM at 50 μg/ml exhibited the highest melanin induction and the activity of tyrosinase of 1.58 and 1.67 folds of the control, respectively, in comparing to other synthesized methyl esters. For the saturated fatty acids, MA at 10.0 μg/ml gave the highest melanin induction and the tyrosinase activity of 1.40 and 1.23 folds of the control, respectively. The observed non-correlation of melanin induction and the tyrosinase activity of most methyl esters and saturated fatty acids may be due to the interaction of the compounds in various steps of the melanogenesis process. The melanogenesis induction of saturated fatty acids has been reported to involve the decreased ubiquitin-proteasome pathway, resulting in the deceleration of tyrosinase degradation. Also, the saturated fatty acid involved in the post-transcriptional regulation, the modification of proteolytic degradation of tyrosinase and other events in melanogenesis process may increase the rate of the pigment biosynthesis. For the methyl esters, the melanogenesis stimulation through the expression of tyrosinase, microphthalmia-associated transcription factor M and tyrosinase-related protein 2 has been demonstrated. Also, the ester has been reported to activate cAMP response element binding (CREB) protein that subsequently increases the melanin production.

4. Since MM showed the highest melanin induction activity with no cytotoxic effect, MM was selected to load in different charged niosomes.

5. Three charged niosomes, including neutral (Brij72/cholesterol at 7:3), cationic (Brij72/cholesterol/ DDAB at 7:3:0.65) and anionic niosomes (Brij72/cholesterol/DP at 7:3:0.65), prepared by chloroform film method with sonication were loaded with MM. The maximum loading and percentage entrapment of MM were 4.5, 90.68$\pm$7.95 in neutral; 11.0, 92.54$\pm$6.32 in cationic and 0.1% w/w, 74.43$\pm$1.86% in anionic niosomes, respectively. The highest loading concentration and percentage of entrapment of MM in cationic niosomes may

be from the charge interaction of the positively charged molecule (DDAB) and the loaded negatively charge compound (MM).

6. All blank and MM loaded niosomes were in unilamellar structures under transmission electron microscope (TEM) and in nanosize at initial and after 3-month storage.

7. The remaining percentages of MM in all niosomal dispersions kept at 4±2, room temperature (30±2) and 45±2°C for 3 months were about 82, 74 and 72% respectively, while the dry MM gave 97.82±1.74, 96.56±2.91 and 91.39±4.32%, respectively. The higher chemical stability of the dry MM than that loaded in niosomes may be from the hydrolysis of MM in the acidic environment (the pH range of all niosomal dispersions was 3-5).

8. The blank neutral, blank cationic and MM loaded in neutral and cationic niosomes showed moderate cytotoxicity in normal human skin fibroblasts at 6^{th} passage and B16F10 melanoma cells at 56.64±3.19, 59.72±1.51; 73.81±2.86, 82.51±0.20; 47.34±2.13, 52.67±2.78 and 73.20±3.49, 84.34±2.75% cell viability, respectively. Blank anionic and MM loaded in anionic niosomes indicated no cytotoxicity in both cell lines. From the cytotoxic ratio of cell viability in normal and cancer cells, all blank and MM loaded in niosomes demonstrated no toxic effect to normal cells.

9. MM loaded in cationic niosomes demonstrated the highest melanin induction with the tyrosinase activity of 1.42 and 1.70 folds of the control and 1.14 and 1.59 folds higher than theophylline, respectively. The higher melanogenesis induction of MM loaded in cationic niosomes than that loaded in other niosomes may be from the enhanced intracellular delivery of the positively charged niosomes in comparing to the neutral and negatively charged niosomes.

10. For transfollicular penetration through porcine skin using follicular closing technique by vertical Franz diffusion cells, MM loaded in cationic niosomes exhibited higher cumulative

amounts of MM in both skin and receiver compartment than that loaded in neutral and anionic niosomes. In fact, the cationic compound treated drug have been demonstrated to enhance the skin permeation of the drug. Thus, the skin permeation of MM may increase when loaded in the cationic charged niosomes.

11. MM loaded in cationic niosomes that indicated the highest melanogenesis induction activity, transfollicular penetration, moderate cytotoxicity and good physicochemical properties was selected to incorporate in the selected hair lotion formulation.

12. The high hydrophilic lipophilic balance (HLB) non-ionic surfactants that gave the changed membrane structure of niosomal vesicles by turbidity and vesicular size evaluation of less than $\pm$15% were selected to use in the hair lotion base formulation. 13. The good physical stability of the base formulation with the positive zeta potential of the formulation (7.21$\pm$0.24 mV) and vesicular size of 237.20$\pm$0.74 nm was chosen to incorporate MM loaded and not loaded in cationic niosomes.

14. The hair lotions containing MM loaded and not loaded in cationic niosomes gave good physical stability when stored at various temperatures (4$\pm$2, 30$\pm$2 and 45$\pm$2°C) for 3 months. The morphology of MM loaded niosomes when incorporated in the hair lotion indicated unilamellar structures under the TEM and in nanosize at initial and after the 3-month storage.

15. The remaining percentages of MM loaded in cationic niosomes were higher than that not loaded in niosomes at all storage temperatures, indicating the environmental protection of MM when loaded in niosomes.

16. Transfollicular penetration by the follicular closing technique using vertical Franz diffusion cells of the hair lotion containing MM loaded in cationic niosomes demonstrated higher cumulative amounts and fluxes than that not loaded in niosomes, due to the effective compound delivery of niosomes.

17. *In vivo* skin irritation by the closed patch test of hair lotions containing MM loaded and not loaded in cationic niosomes demonstrated slight irritation which was in the agreement with the above cytotoxicity assay *in vitro*.

18. For *in vivo* melanogenesis induction in aged mice, the peak of the skin and hair pigmentation was first observed in hair lotions containing MM loaded in cationic niosomes, followed by hair lotions containing MM not loaded in cationic niosomes and theophylline (the positive control), respectively. Also, the histological examination using melanin bleach staining technique exhibited the alterations in skin, melanization and hair follicle formation in the mice treated with the hair lotions containing MM loaded and not loaded in cationic niosomes and theophylline.

In summary, MM which was synthesized by Fischer esterification exhibited the highest melanogenesis induction activity without cytotoxicity and appeared to be appropriate to develop for canities treatment. The cationic niosomes loaded with MM demonstrated the good physicochemical properties for the 3-month storage at 4$\pm$2, 30$\pm$2 and 45$\pm$2°C as well as the highest *in vitro* melanin induction activity and transfollicular penetration. The *in vivo* rabbit skin irritation of the hair lotion consisted of cationic niosomes loaded with MM demonstrated slight irritation. For *in vivo* melanogenesis induction in the aged mice, the peak of the skin and hair pigmentation was first observed in hair lotion containing cationic niosomes loaded with MM, indicating the enhanced skin penetration of MM loaded in cationic niosomes. The alterations in skin, melanization and hair follicle formation in the histological examination of the skin treated with hair lotion containing cationic niosomes loaded with MM using the melanin bleach staining technique was observed. Hence, the hair lotion containing cationic niosomes loaded with MM is expected to be used for the treatment of canities at the early stage.

For the further research, the hair lotion containing cationic niosomes loaded with MM should be further investigated for hair pigmentation activity in human volunteers. Also, the preparation of MM in this study may be difficult for scaling up in the commercial production. Besides, the addition of UV or IR radiation together with topical treatment may be useful to shorten the treatment time of production application in the *in vivo* evaluation. Generally, UV radiation is used in a combined therapy with the pigment inducing substances to treat the skin pigment formation disorders, due to its pigment stimulating potential in pigment producing cells. For IR radiation, it has been shown to increase the permeation of topically applied compound through the enhanced blood flow.