CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of saturated fatty acid methyl esters

3.1.1 Synthesis of saturated fatty acid methyl esters

The methyl ester derivatives of four saturated fatty acids were synthesized by Fischer esterification and used acid as a catalyst. Although each step of the reaction was reversible, the excess addition of methanol drove the equilibrium reaction to obtain the required ester products[56]. Also, the reaction conditions gave the reasonable yields of all methyl esters. Table 10 showed the percentage yields, physical characteristics and identification data of saturated fatty acid methyl ester derivatives by FTIR and GC/MS. Physical characteristics of the three synthesized esters, including lauric acid methyl ester (LM), myristic acid methyl ester (MM) and palmitic acid methyl ester (PM), were clear, colorless, odorless and oily liquids at room temperature (30$\pm$2°C), whereas stearic acid methyl ester (SM) was in white solid. Analysis of these synthesized esters by FTIR and GC/MS was performed in comparing to their corresponding saturated fatty acids. Figure 28 showed the FTIR spectra of MA and MM. Figure 29 demonstrated the GC/MS spectra of PM in comparing to the standard palmitic acid methyl ester. Both spectra from FTIR and GC/MS indicated that all synthesized compounds were methyl ester derivatives of their corresponding saturated fatty acids (LA, MA, PA and SA).

3.1.2 Physical and chemical stability of saturated fatty acid methyl esters

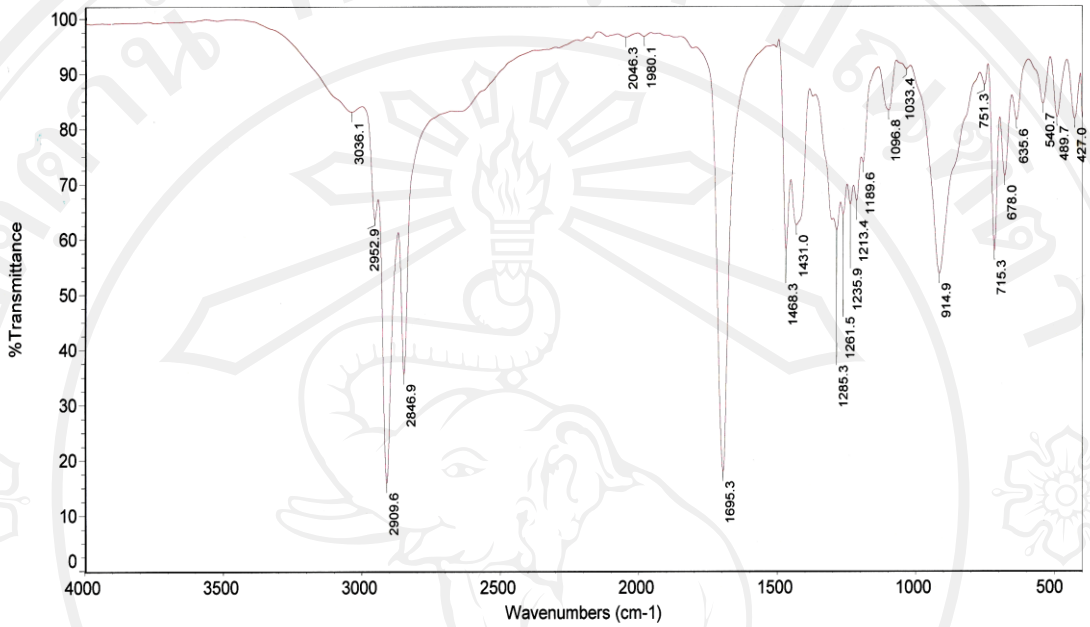
For stability evaluation, the dry form of four methyl esters kept in vials was

Table 10 Percentage yields, physical characteristics and identification data of saturated fatty acid methyl ester derivatives

Compounds	Percentage yields (%)	Physical characteristics	Melting point (°C)	Boiling point (°C)	pH*	FTIR spectra (cm^{-1})	GC/MS	
							retention time (min)	Content (%w/w)
Lauric acid methyl ester (LM)	61.37	clear, colorless, odorless and oily liquid	6-10	232-234	6.0	2922.1, 2853.0, 1740.5, 1461.1, 1436.6, 1360.6, 1167.3, 722.5	22.96	98.21
Myristic acid methyl ester (MM)	68.06	clear, colorless, odorless and oily liquid	17-19	270-272	6.0	2921.4, 2852.5, 1740.9, 1460.5, 1438.2, 1360.5, 1167.5, 722.3	29.41	99.18
Palmitic acid methyl ester (PM)	43.22	clear, colorless, odorless and oily liquid	29-31	290-292	5.8	2914.6, 2847.3, 1738.4, 1463.6, 1434.3, 1374.3, 1158.4, 724.0	36.26	99.34
Stearic acid methyl ester (SM)	54.64	white solid	38-40	300-302	5.8	2913.5, 2846.4, 1737.0, 1462.2, 1434.6, 1376.8, 1164.6, 721.7	42.45	99.39

*pH of the saturated fatty acid methyl ester at 10 mg/ml in ethanol at room temperature (30±2°C)

(a) MA



(b) MM

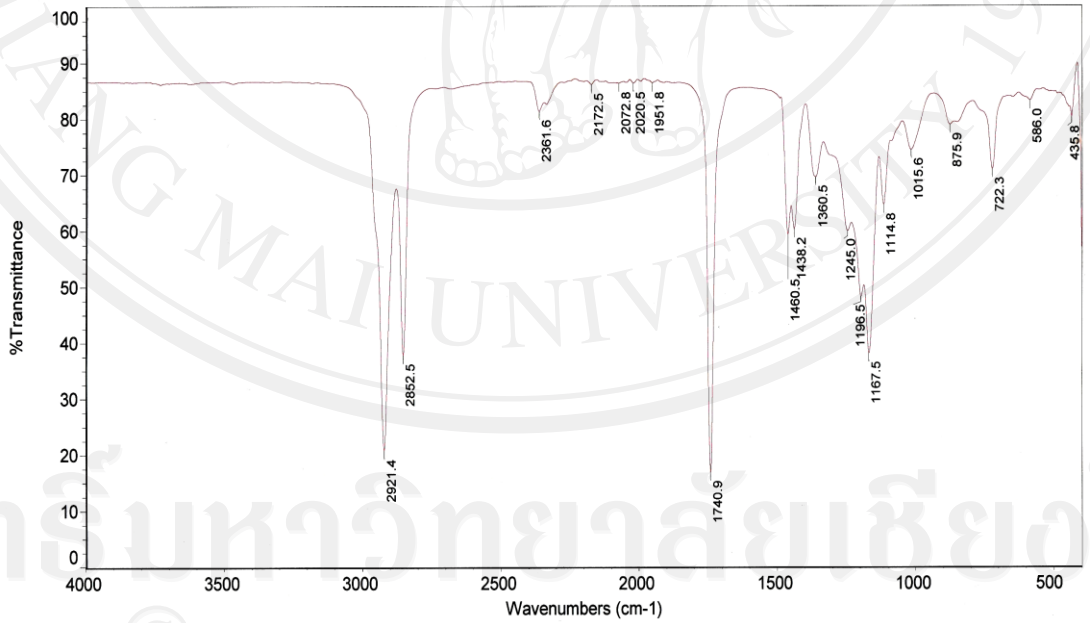
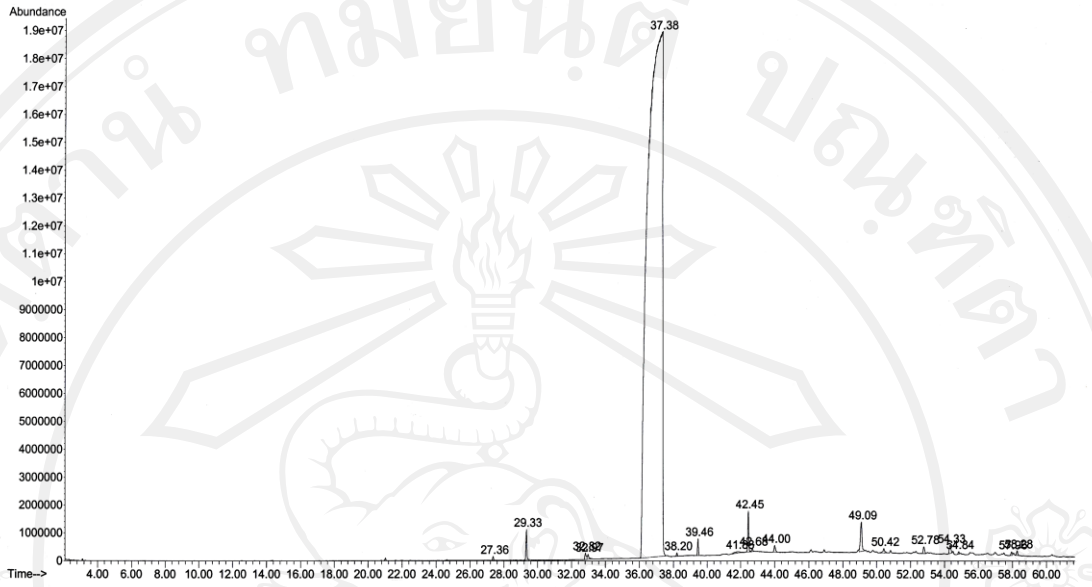


Figure 28 FTIR spectra of myristic acid (MA; a) and myristic acid methyl ester (MM; b)

(a) standard PM



(b) synthesized PM

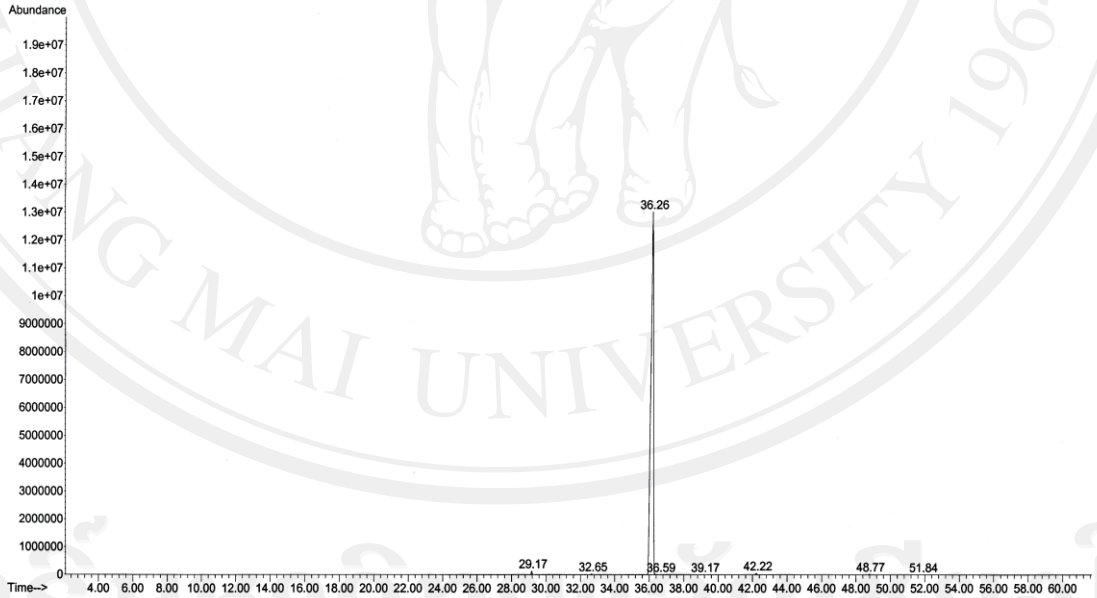


Figure 29 GC/MS spectra of standard palmitic acid methyl ester (standard PM; a) and synthesized palmitic acid methyl ester (PM; b)

stored at 4±2, room temperature (30±2) and 45±2°C for 3 months. The physical characteristics of all methyl esters did not change, whereas the chemical stability demonstrated the decreased ester contents in comparing to at initial (Fig. 30). Since higher temperature could accelerate the degradation of the compound, the decreased rate of all methyl ester contents kept at high temperature has been reported to be faster than at low temperature[56].

3.2 *In vitro* cell cytotoxicity and melanogenesis induction assays of the saturated fatty acid methyl esters

3.2.1 Cell cytotoxicity by SRB assay

Theophylline at 2.5, 5.0 and 10.0 μg/ml increased cell proliferation higher than the control (Fig. 31), while the saturated fatty acid methyl esters and their corresponding saturated fatty acids demonstrated the increased cytotoxic effect with increased concentrations (Fig. 32). LA and MA at 2.5 – 25.0 μg/ml showed no effect on cytotoxicity. However, LA and MA at 50.0 μg/ml slightly decreased cell viability. For longer chain saturated fatty acids, the cytotoxic effect of PA and SA depended on concentrations. At low concentrations, cell viability was high. The increased concentrations decreased the cell survival. LM and MM at all concentrations showed no effect on cell cytotoxicity. But, PM and SM suppressed cell viability depending on concentrations. For the comparison of saturated fatty acids and methyl ester derivatives on cytotoxicity, only PA and PM significantly demonstrated different effect at all concentrations (p<0.01). The higher cytotoxicity of PA and SA (16 and 18 carbons) than that of LA and MA (12 and 14 carbons) may be due to their longer carbon chain, resulting in the higher lipophilicity and lipid solubility than the shorter

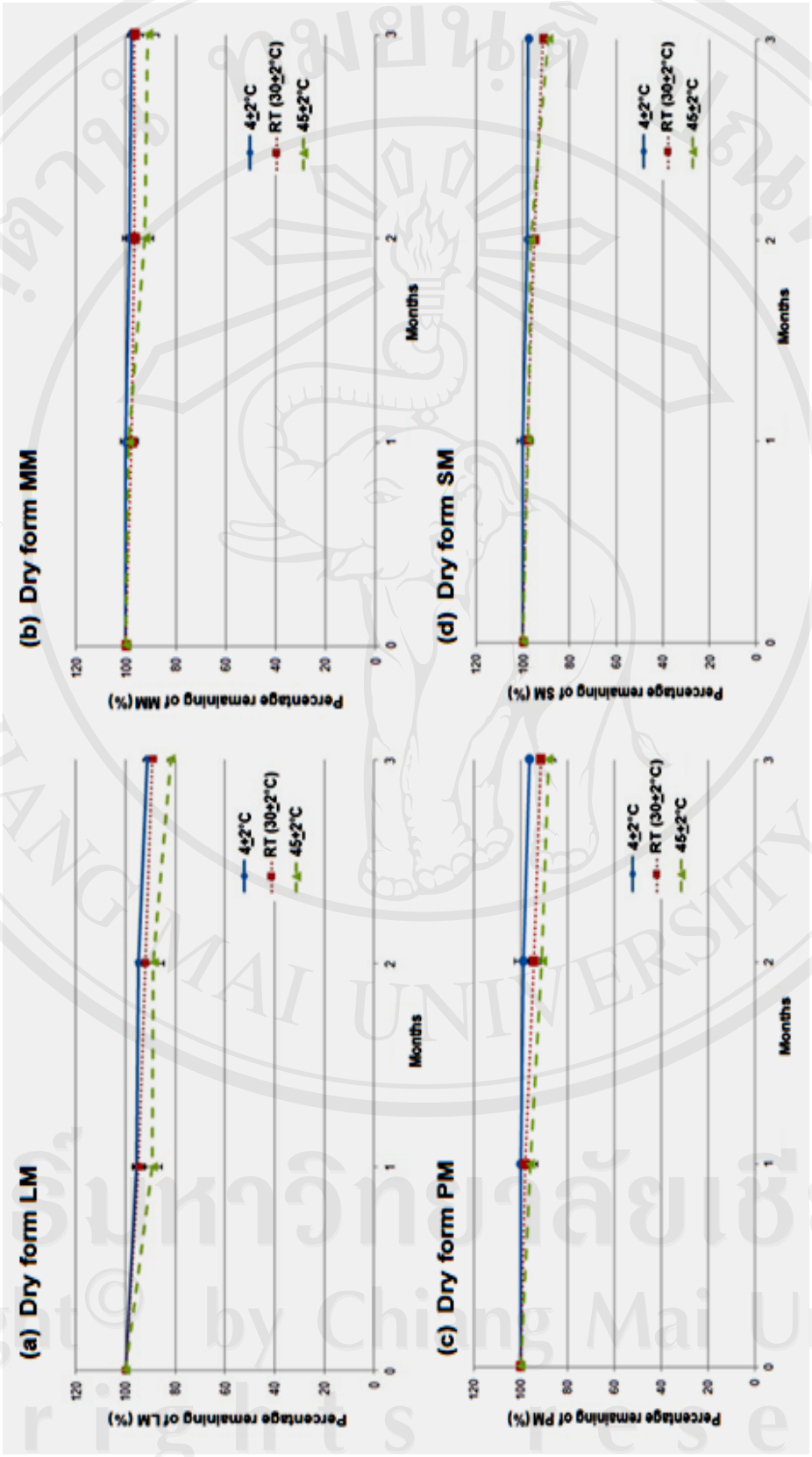


Figure 30 Percentage remaining of lauric acid methyl ester (LM; a), myristic acid methyl ester (MM; b), palmitic acid methyl ester (PM; c) and stearic acid methyl ester (SM; d)

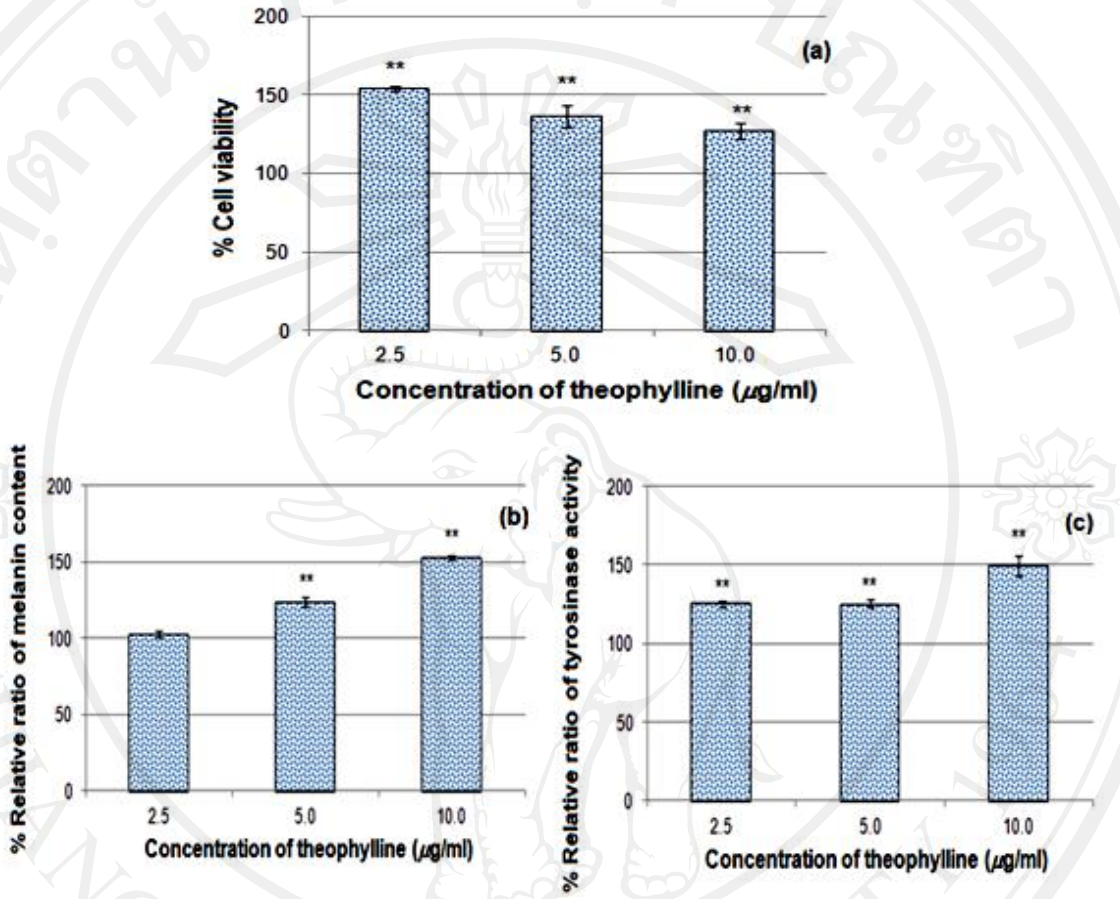


Figure 31 Percentage cell viability (a), percentage relative ratio of melanin content (b) and percentage relative ratio of tyrosinase activity (c) of B16F10 melanoma cells treated with theophylline, a positive control, at 2.5, 5.0 and 10.0 μg/ml. *Significant difference from the control (*p<0.05, **p<0.01)

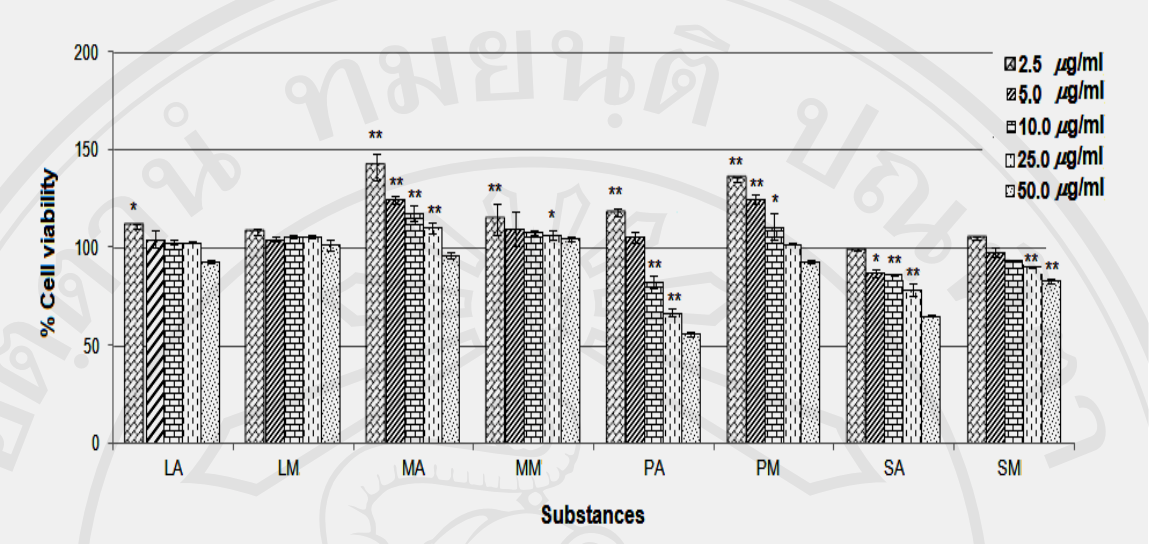


Figure 32 Percentage cell viability of B16F10 melanoma cells treated with various concentrations of esters [lauric acid methyl ester (LM), myristic acid methyl ester (MM), palmitic acid methyl ester (PM) and stearic acid methyl ester (SM)] and their corresponding saturated fatty acids [lauric acid (LA), myristic acid (MA), palmitic acid (PA) and stearic acid (SA)]. Data represented mean ± S.E. of three independent experiments. *Significant difference from the control (*p<0.05, **p<0.01)

chain[45]. Also, saturated fatty acids with longer hydrocarbon chain may penetrate into the cell membrane composing of the lipid soluble constituents, such as phospholipids, ceramides and cholesterol, more efficient than those with the shorter hydrocarbon chain, thereby reducing cell viability[205]. Meanwhile, the methyl esters at all concentrations (2.5-50.0 μg/ml) demonstrated more than 80% viable cells and showed less cytotoxic effect than their corresponding saturated fatty acids. This may be due to the less acidity of the esters than their parent saturated fatty acids[56]. In addition, this result was in agreement with the previous report[7].

3.2.2 Melanin content measurement

For evaluation of melanogenesis induction, theophylline, a melanogenesis stimulator, was used as a reference. This pigment stimulator regulates the biosynthesis through cAMP pathway as well as increases the gamma-glutamyl transpeptidase- and tyrosinase-reactive cells, resulting in the induction of melanin production with the increased level of tyrosinase activity[206-207]. Theophylline at 2.5-10.0 μg/ml was evaluated in our preliminary study. The melanin production as well as the tyrosinase activity increased with increased concentrations (Fig. 31). This correlation agreed with the previous reports[208-209].

The effects of saturated fatty acids and methyl esters on melanin production were shown in Fig. 33. All saturated fatty acids and their methyl esters demonstrated different melanin induction. Among the saturated fatty acids, MA at 10.0 μg/ml showed the highest melanin content at 1.40 folds of the control. For the highest melanin content of LA at 50.0 μg/ml, PA at 5.0 μg/ml and SA at 10.0 μg/ml were 1.09, 1.25 and 1.22 folds of the control, respectively. In the methyl ester-treated group, MM demonstrated the melanogenesis stimulation in the concentration-dependent manner. Also, MM at 50.0 μg/ml exhibited the highest melanin induction of 1.58 folds of the control, which was almost the same value as theophylline (1.53 folds of the control). For other three esters at 10.0 μg/ml, the highest melanin induction of LM, PM and SM were 1.18, 1.18 and 1.24 folds of the control, respectively. In comparison of the saturated fatty acids and their methyl esters, LM at 2.5, 10.0 and 25.0 μg/ml significantly exhibited higher melanin content than LA. MM at 25.0 and 50.0 μg/ml significantly demonstrated higher melanin content than MA. PA at 5 μg/ml significantly gave higher melanin content than PM. However,

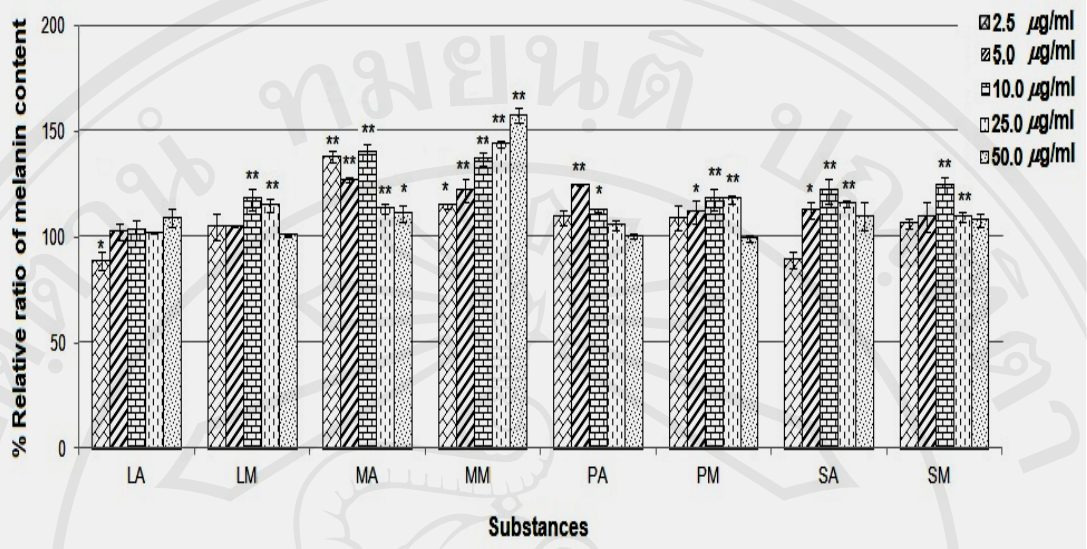


Figure 33 Percentage relative ratio of melanin content of B16F10 melanoma cells treated with various concentrations of esters [lauric acid methyl ester (LM), myristic acid methyl ester (MM), palmitic acid methyl ester (PM) and stearic acid methyl ester (SM)] and their corresponding saturated fatty acids [lauric acid (LA), myristic acid (MA), palmitic acid (PA) and stearic acid (SA)]. Data represented mean $\pm$ S.E. of three independent experiments. *Significant difference from the control (*p<0.05, **p<0.01)

PM at 25.0 μg/ml significantly induced melanogenesis higher than its parent saturated fatty acid (PA). SM at 2.5 μg/ml demonstrated melanin induction higher than SA. The lowest melanin induction was observed in LA, owing to the lowest lipophilicity in comparing with other three saturated fatty acids. The low lipophilic compounds may penetrate into the cell membranes (which have various organelles and nucleus inside) less effective than the high lipophilic compounds. Thus, the higher lipophilic saturated fatty acids including MA, PA and SA may penetrate into the cell membrane and induce the melanin production better than LA. However, melanin induction

potential of the saturated fatty acids appeared to have limitation. The increased concentrations of the saturated fatty acids demonstrated the decreased induction. This may be from the more acidity of the compounds when their concentrations increase. PA and SA have the acid dissociation constant values (pKa) lower than MA, thereby being more acidic than MA[56]. The more acidity may not be the optimal pH for pigment production that subsequently decrease the melanin content[210]. The pH of MA and ME solutions at 10 mg/ml in ethanol were 5.0 and 6.0, respectively. This indicated that the methyl ester obtained from the esterification reaction between the saturated fatty acid and methanol was higher lipophilic and lower acidic than the parent saturated fatty acid. Thus, the higher lipophilicity and lower acidity of LM may facilitate the penetration across the cell membrane, resulting in the higher melanin induction than LA. Also, the correlation between the concentration and the melanin induction was observed in MM. This may be from the proper compound lipophilicity for cell penetration in the combination with the appropriate pH environment for melanin induction. PM at 2.5 and 5.0 μg/ml demonstrated lower melanin stimulation than PA, its corresponding saturated fatty acid. This data was similar to the previous study that indicated the more effective of PA on melanogenesis activity than PM[7]. When the concentrations increased, PM exhibited the pigment induction higher than PA. SM at 2.5 μg/ml demonstrated higher melanin induction than SA. However, when the SM concentrations increased, the less pigment induction than its corresponding saturated fatty acid was observed in similar to PM and PA. Hence, the highest melanogenesis induction of MA and MM (14-carbon saturated fatty acid and methyl ester) may be from their proper lipophilicity that may be similar to the major structural components of cell membrane in the combination

with the appropriate pH circumstance for melanin production[211].

3.2.3 Tyrosinase activity measurement

The increased concentration of theophylline, a positive control, indicated the increased tyrosinase activity (Fig. 31). Among the saturated fatty acids, the highest tyrosinase activities of LA, MA, PA and SA were 1.14, 1.23, 1.03 and 1.50 folds of the control, respectively. For the esters, LM, MM, PM and SM showed 1.23, 1.67, 1.22 and 1.13 folds of the control, respectively (Fig. 34). Tyrosinase activity of most saturated fatty acids and methyl esters did not relate to their melanogenesis induction activity. This can be explained that pigment biosynthesis in melanocytes involves various steps and the pigment stimulation compounds can act on many steps of the process. The melanogenesis induction of the saturated fatty acid has been reported on the involvement of the decreased ubiquitin-proteasome pathway that leads to the deceleration of tyrosinase degradation[5,42,45]. In addition, various actions of the compound on melanogenesis process including post-transcriptional regulation, the modification of proteolytic degradation of tyrosinase and other events, may establish the rate of pigment biosynthesis[6, 42, 212-213]. For the methyl ester, the melanogenesis stimulation via the expression of tyrosinase, microphthalmia-associated transcription factor M (MITF-M) and tyrosinase-related protein-2 (TRP-2) proteins have been demonstrated. Also, the ester has been reported to activate cAMP response element binding (CREB) protein, that subsequently increases the melanogenesis[7]. Thus, the observed non-correlation of tyrosinase and melanogenesis activity of the saturated fatty acids and their derivatives may be from the above difference and the complex mechanism of actions of the compounds.

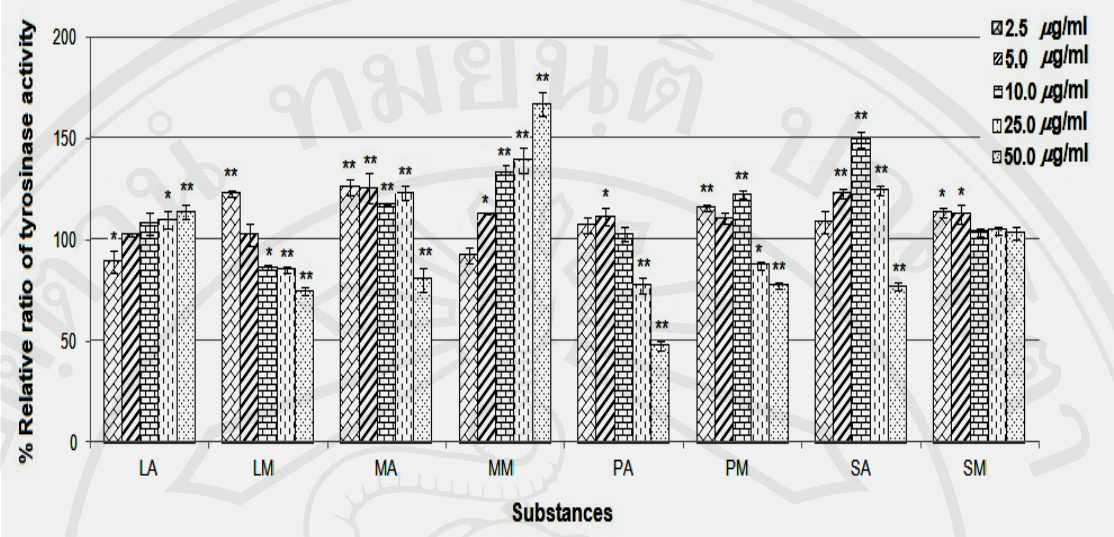


Figure 34 Percentage relative ratio of tyrosinase activity of B16F10 melanoma cells treated with various concentrations of esters [lauric acid methyl ester (LM), myristic acid methyl ester (MM), palmitic acid methyl ester (PM) and stearic acid methyl ester (SM)] and their corresponding saturated fatty acids [lauric acid (LA), myristic acid (MA), palmitic acid (PA) and stearic acid (SA)]. Data represented mean ± S.E. of three independent experiments. *Significant difference from the control (*p<0.05, **p<0.01)

3.2.4 Selection of the saturated fatty acid methyl ester to load in the selected blank niosomes

The methyl esters of four saturated fatty acids were synthesized and evaluated for their cytotoxicity and melanogenesis induction activity in comparing to their corresponding saturated fatty acids. Myristic acid methyl ester (MM) that showed the highest melanin induction activity with no cytotoxic effect was selected to load in different charged niosomes. In addition, MM was repeatedly synthesized via the Fischer esterrification as well as identification by GC/MS to obtain the more

amounts of purified ester for the further study. The synthesized MM was clear and oily liquid at room temperature (30±2°C) and obtained from the esterification reaction of myristic acid and methanol at the 1:5 molar ratio. The percentage yield was 50.36% and the purity was 98.34% at the retention time of 30.39 min (Fig. 35).

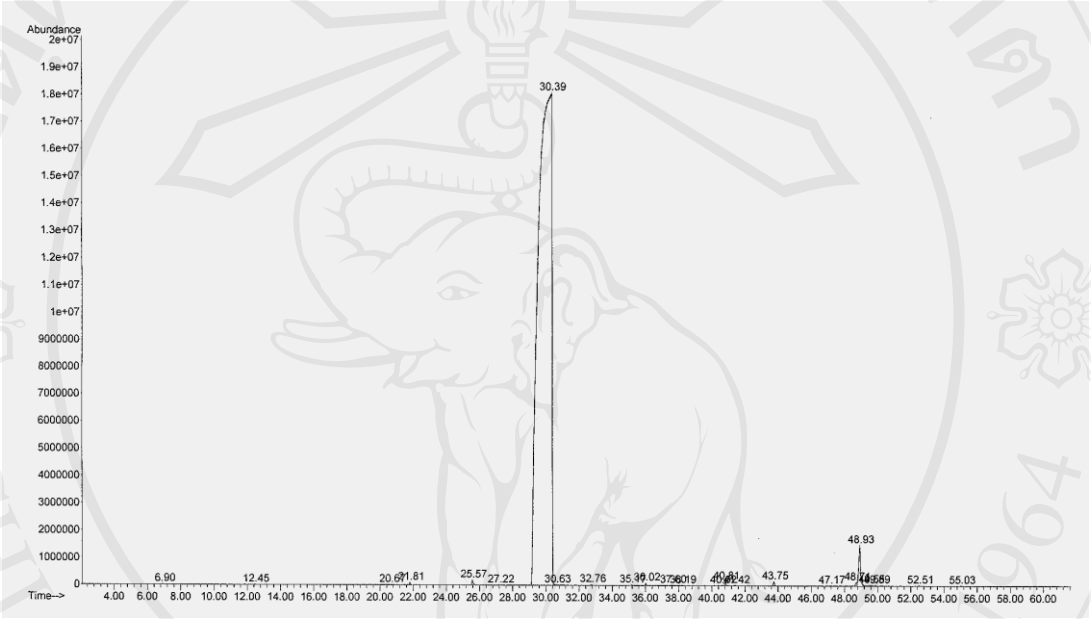


Figure 35 GC/MS spectrum of myristic acid methyl ester (MM)

3.3 Development of the blank niosomes

3.3.1 Physical characteristics of the blank niosomes

Twelve blank niosomes with three different charges were prepared and evaluated for their physical characteristics at initial and 3-month storage at 4±2, room temperature (30±2) and 45±2°C. Tables 11-13 showed the physical characteristics of blank neutral, cationic and anionic niosomes, respectively. At initial, all neutral blank niosomal formulations exhibited the negative zeta potential values of -50.87±0.62, -44.87±0.49, -39.47±1.11 and -12.77±0.12 mV and the average size of 409.37±7.31, 99.42±0.04, 93.36±1.10 and 144.23±2.81 nm were for Span60/cholesterol,

Tween61/cholesterol at 1:1, Tween61/cholesterol at 7:3 and Brij72/cholesterol, respectively. The sedimentation of Span60/cholesterol vesicles was detected at all temperatures after 2 months. After 3 months, the increased negative zeta potential of vesicles composing of Span60/cholesterol and Brij72/cholesterol were observed, whereas that of Tween61/cholesterol vesicles at both molar ratio decreased. However, the zeta potential of all neutral blank vesicles indicated the less aggregation after 3-month storage. The vesicular size of neutral blank vesicles was in the nanosize range at initial and after 3 months.

For the cationic and anionic blank niosomes, vesicles composing of Tween61/cholesterol and charged molecules at 1:1:0.3 and 7:3:1.5 molar ratio demonstrated larger vesicular size than the neutral niosomes. But, Brij72/cholesterol cationic and anionic niosomes gave smaller size than the neutral niosomes. Meanwhile, the order of the largest to the smallest size of niosomes composing of Span60/cholesterol system was cationic, neutral and anionic niosomes. The different vesicular size of niosomal formulations composing of different non-ionic surfactant systems may be from the non-ionic surfactant, including the HLB values and the geometrical shape of the surfactants, as well as the addition of the charged molecule and the charge repulsion and attraction in the vesicular formation[73,214-215]. During the hydration process, types of net charge developed within the bilayer membranes before the formation of vesicle may affect the rigidity of the membrane, the rate of curving, splitting and vesicle forming, resulting in the changed vesicular size[216]. After 3-month storage of cationic blank niosomes, the slight change in vesicular size and polydispersity index of the vesicles composing of Tween61/ cholesterol/DDAB at 1:1:0.3 and Brij72/cholesterol/DDAB were observed, whereas the size of Span60/

Table 11 Characteristics of blank neutral niosomes prepared by chloroform film method with sonication when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

System	Molar ratio	pH		Z-zeta potential (mV)		Particle diameter (nm)		Polydispersity index	
		Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
Span60:Cholesterol									
45±2°C	7:3	4.0	4.0	-50.87±0.62	-75.80±0.61	409.37±7.31	522.73±2.92	0.49±0.01	0.58±0.03
RT (30±2°C)	7:3	4.0	5.0	-50.87±0.62	-51.33±1.19	409.37±7.31	407.47±20.61	0.49±0.01	0.57±0.04
4±2°C	7:3	4.0	5.0	-50.87±0.62	-45.87±0.70	409.37±7.31	264.37±4.85	0.49±0.01	0.63±0.03
Tween61:Cholesterol									
45±2°C	1:1	4.0	4.0	-44.87±0.49	-34.67±0.58	99.42±0.04	110.30±0.87	0.24±0.00	0.12±0.02
RT (30±2°C)	1:1	4.0	4.0	-44.87±0.49	-38.13±0.32	99.42±0.04	92.93±0.11	0.24±0.00	0.21±0.01
4±2°C	1:1	4.0	5.0	-44.87±0.49	-38.60±0.78	99.42±0.04	95.26±0.20	0.24±0.00	0.23±0.00
Tween61:Cholesterol									
45±2°C	7:3	4.0	4.0	-39.47±1.11	-32.80±2.06	93.36±1.10	216.13±1.19	0.24±0.00	0.15±0.01
RT (30±2°C)	7:3	4.0	4.0	-39.47±1.11	-36.50±0.95	93.36±1.10	139.80±0.53	0.24±0.00	0.51±0.00
4±2°C	7:3	4.0	5.5	-39.47±1.11	-37.63±0.98	93.36±1.10	96.16±0.35	0.24±0.00	0.23±0.01
Brij72:Cholesterol									
45±2°C	7:3	5.0	5.0	-12.77±0.12	-25.60±0.12	144.23±2.81	170.97±1.44	0.18±0.01	0.21±0.01
RT (30±2°C)	7:3	5.0	5.0	-12.77±0.12	-36.60±0.45	144.23±2.81	175.53±0.58	0.18±0.01	0.26±0.02
4±2°C	7:3	5.0	5.0	-12.77±0.12	-34.87±1.00	144.23±2.81	190.47±1.25	0.18±0.01	0.34±0.02

Table 12 Characteristics of blank cationic niosomes prepared by chloroform film method with sonication when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

System	Molar ratio	pH		Z-zeta potential (mV)		Particle diameter (nm)		Polydispersity index	
		Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
Span60:Cholesterol:DDAB									
45±2°C	7:3:0.75	4.0	3.0	63.50±1.01	45.97±1.89	782.40±19.85	534.70±28.86	0.68±0.03	0.52±0.02
RT (30±2°C)	7:3:0.75	4.0	4.0	63.50±1.01	44.23±0.43	782.40±19.85	338.90±10.97	0.68±0.03	0.58±0.05
4±2°C	7:3:0.75	4.0	4.0	63.50±1.01	55.23±1.32	782.40±19.85	223.33±2.31	0.68±0.03	0.36±0.01
Tween61:Cholesterol:DDAB									
45±2°C	1:1:0.3	4.0	3.0	59.57±1.93	54.50±0.76	103.67±0.24	103.67±1.89	0.24±0.00	0.25±0.01
RT (30±2°C)	1:1:0.3	4.0	3.0	59.57±1.93	52.90±1.85	103.67±0.24	123.77±0.23	0.24±0.00	0.28±0.02
4±2°C	1:1:0.3	4.0	4.0	59.57±1.93	61.67±0.64	103.67±0.24	101.35±1.03	0.24±0.00	0.24±0.01
Tween61:Cholesterol:DDAB									
45±2°C	7:3:1.5	4.0	3.0	47.27±4.44	46.57±0.83	99.55±0.56	174.53±2.11	0.21±0.00	0.73±0.02
RT (30±2°C)	7:3:1.5	4.0	3.0	47.27±4.44	49.60±0.52	99.55±0.56	258.97±1.83	0.21±0.00	0.51±0.03
4±2°C	7:3:1.5	4.0	4.0	47.27±4.44	49.20±2.61	99.55±0.56	101.90±0.17	0.21±0.00	0.24±0.01
Brij72:Cholesterol:DDAB									
45±2°C	7:3:0.65	5.0	5.0	59.77±0.70	53.67±3.20	112.70±0.84	130.33±1.65	0.21±0.02	0.24±0.02
RT (30±2°C)	7:3:0.65	5.0	5.0	59.77±0.70	56.83±0.84	112.70±0.84	125.77±1.65	0.21±0.02	0.23±0.01
4±2°C	7:3:0.65	5.0	5.0	59.77±0.70	55.07±1.11	112.70±0.84	121.23±0.98	0.21±0.02	0.25±0.01

Table 13 Characteristics of blank anionic niosomes prepared by chloroform film method with sonication when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

System	Molar ratio	pH		Z-zeta potential (mV)		Particle diameter (nm)		Polydispersity index	
		Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
Span60:Cholesterol:DP									
45±2°C	7:3:0.75	4.0	4.0	-63.27±0.44	-54.50±0.95	201.80±4.36	263.97±0.73	0.44±0.01	0.41±0.01
RT (30±2°C)	7:3:0.75	4.0	4.0	-63.27±0.44	-55.57±0.74	201.80±4.36	621.50±25.06	0.44±0.01	0.79±0.04
4±2°C	7:3:0.75	4.0	4.0	-63.27±0.44	-57.87±0.15	201.80±4.36	264.43±3.93	0.44±0.01	0.54±0.04
Tween61:Cholesterol:DP									
45±2°C	1:1:0.3	4.0	3.0	-58.40±0.25	-48.93±0.32	125.07±1.92	186.73±2.68	0.34±0.01	0.48±0.03
RT (30±2°C)	1:1:0.3	4.0	3.0	-58.40±0.25	N/A	125.07±1.92	N/A	0.34±0.01	N/A
4±2°C	1:1:0.3	4.0	4.0	-58.40±0.25	-32.77±0.26	125.07±1.92	134.90±6.64	0.34±0.01	0.53±0.01
Tween61:Cholesterol:DP									
45±2°C	7:3:1.5	4.0	3.0	-56.80±0.61	-48.57±1.94	179.57±4.15	210.70±7.32	0.47±0.04	0.60±0.04
RT (30±2°C)	7:3:1.5	4.0	3.0	-56.80±0.61	-53.40±1.07	179.57±4.15	1070.27±56.05	0.47±0.04	0.78±0.04
4±2°C	7:3:1.5	4.0	4.0	-56.80±0.61	-34.53±0.44	179.57±4.15	161.50+12.72	0.47±0.04	0.60±0.05
Brij72:Cholesterol:DP									
45±2°C	7:3:0.65	4.0	4.0	-57.73±2.19	-45.53±0.09	118.40±0.66	133.00±0.26	0.32±0.00	0.29±0.01
RT (30±2°C)	7:3:0.65	4.0	3.0	-57.73±2.19	-48.13±0.52	118.40±0.66	147.60±2.06	0.32±0.00	0.31±0.02
4±2°C	7:3:0.65	4.0	4.0	-57.73±2.19	-34.40±1.39	118.40±0.66	156.90±1.47	0.32±0.00	0.45±0.03

cholesterol/DDAB and Tween61/cholesterol/DDAB at 7:3:1.5 vesicles showed significant change both in vesicular size and polydispersity index, especially at the high storage temperatures (room temperature and 45±2°C). For the anionic niosomes, the sedimentation and microbial contamination of vesicles prepared from Tween61/cholesterol/DP at 1:1:0.3 were detected after the 2-month storage at room temperature. This system was then discarded. The sedimentation of anionic blank niosomes was also found in Span60/cholesterol/DDAB and Tween61/ cholesterol/ DDAB at the molar ratio of 7:3:1.5.

3.3.2 Selection of the blank niosomes to load with the selected saturated fatty acid methyl ester

The three blank niosomal systems prepared from Brij72/cholesterol with and without the charged molecules (DDAB and DP) exhibited the high physical stability with the nanosize range in comparing to the other three systems, including Span60/cholesterol, Tween61/cholesterol at 1:1 and 7:3 with and without the charged molecules. Thus, these three charged blank niosomal systems were selected to load with MM for the further study.

3.4 Development of the selected saturated fatty acid methyl ester loaded in the selected niosomes

3.4.1 Maximum loading of myristic acid methyl ester or methyl myristate (MM) in the selected niosomes

The three blank niosomal formulations consisting of Brij72/cholesterol with and without the positively charged molecule (DDAB) or the negatively charged molecule (DP) were selected to load with MM due to their high physical stability with

the nanosize range of 120-200 nm and the zeta potential values of -12.77±0.12, 59.77±0.70 and -57.73±2.19 mV for neutral, cationic and anionic niosomes, respectively (Tables 11-13).

For loading MM in the three selected niosomal formulations, the white precipitation was observed at the MM concentrations more than 4.5, 11.0 and 0.1% w/w for neutral, cationic and anionic niosomes, respectively. The loading capacities of MM ranging from the highest to the lowest were cationic, neutral and anionic niosomes. The loading concentration of MM in anionic niosomes was low. This may be owing to the repulsive steric or electrostatic effect of the niosomes and the negatively charged MM (MM suspended in an aqueous solution gave the zeta potential value of -37.10±1.39 mV). Cholesterol, which is usually added in the niosomal formulation, stabilizes the vesicular membrane to be more rigid in protecting the entrapped solute not to be leaked out from the niosomes. As known, DDAB and DP, the charged molecules, can enhance the loading capacity of the negatively and positively charged substances, respectively, by charge interaction. Besides loading in the vesicles, the charged molecules can also be adsorbed on the vesicular membranes. The compounds which have the same charge as the niosomal membrane would expect to be less loaded in the vesicles than those with the opposite charge to the vesicles[71,217-218].

3.4.2 Entrapment efficiency of MM at the maximum loading concentrations in the selected niosomes

The percentages of entrapment at the maximum loading of MM in neutral, cationic and anionic niosomes were 90.68±7.95, 92.54±6.32 and 74.43±1.86%, respectively. As known, the entrapment efficiency of the compound in the close

bilayer vesicles depends on the compositions and types of the vesicles. Also, at the niosomal solid interface, the uneven charge distribution of the loaded compounds and the charged molecules in the niosomal compositions can affect the rate of entrapment of the compound in niosomes[218]. The cationic niosomes gave the highest entrapment efficiency of MM. Similarly to the maximum loading of MM, the charge on the niosomal membrane and MM played an important role to give this effect.

3.4.3 Morphology of the selected niosomes loaded with MM

Figure 36 showed the TEM images of blank and niosomes loaded with MM loaded in using the negative staining technique. All blank niosomes and niosomes loaded with MM indicated unilamellar vesicular structure with the spherical shape. The TEM images of some niosomal vesicles that showed the elongated shape may be from the conformation change of the niosomal membrane affected by the neighboring niosomal vesicles[219].

3.4.4 Physical stability of the selected niosomes loaded with MM

The physical characteristics of the blank niosomes and niosomes loaded with MM were shown in Table 14. At initial, all vesicular systems were in the nanosize range of 112 – 155 nm. The pH values of neutral, cationic and anionic niosomes loaded with MM were 5.0, 4.8 and 4.0, respectively. Blank cationic and anionic niosomes indicated the smaller vesicular size than the blank neutral niosomes. The presence of the charged molecule in the bilayers generally increases the aqueous compartment volume due to the charge repulsion between the ionized head group, which in turn increases the particle size[214-215]. However, some studies have also reported that the inclusion of the charged molecule tends to reduce the vesicular size[74-75]. Since cationic and anionic niosomes loaded with MM gave the smaller

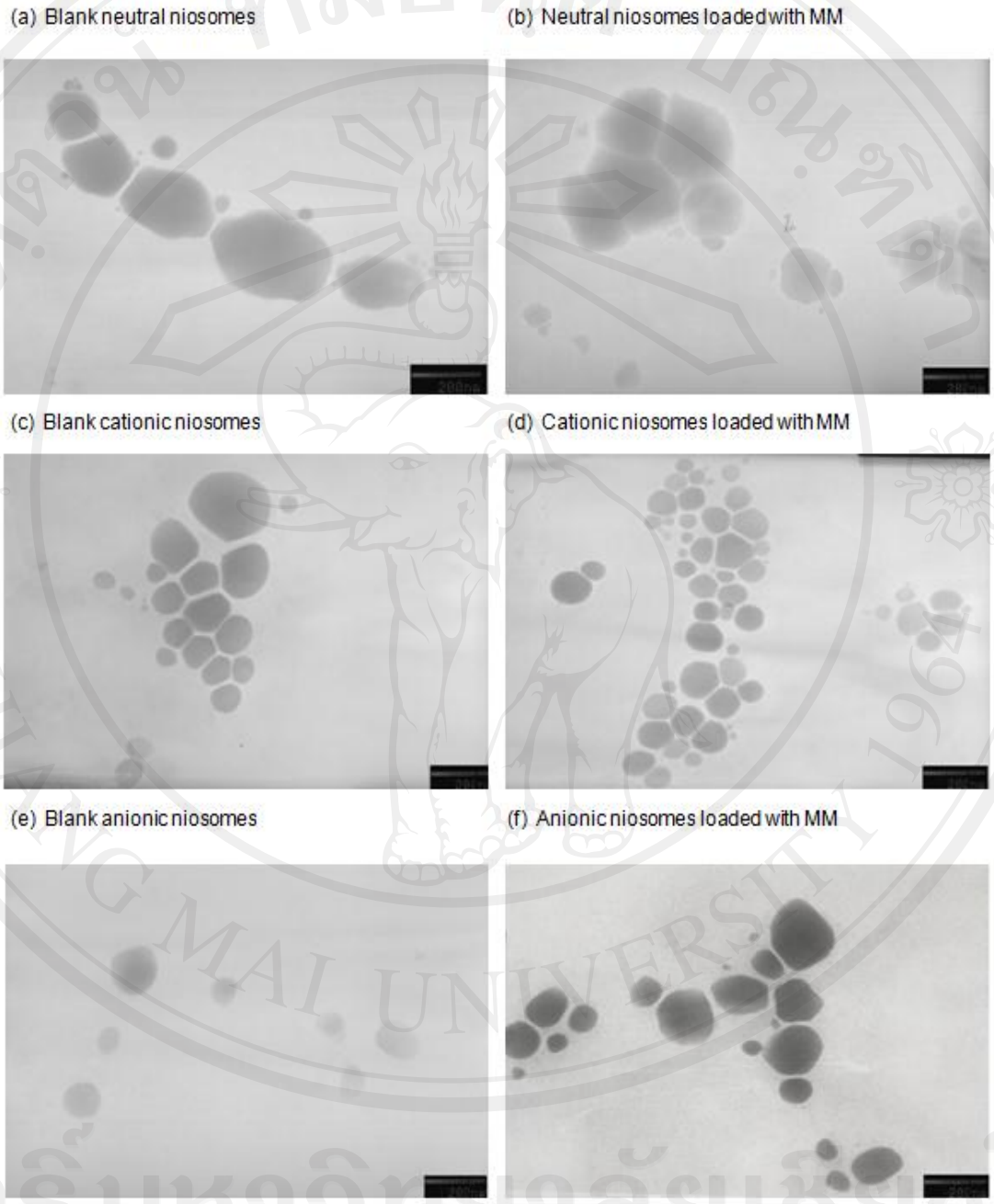


Figure 36 The negative staining TEM images of blank neutral niosomes (a), neutral niosomes loaded with MM (b), blank cationic niosomes (c), cationic niosomes loaded with MM (d), blank anionic niosomes (e) and anionic niosomes loaded with MM (f).

Table 14 Characteristics of various charged niosomes loaded with MM prepared by chloroform film method with sonication when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

System	pH		Z-zeta potential (mV)		Particle diameter (nm)		Polydispersity index	
	Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
Neutral niosomes								
- blank neutral niosomes	5.0	5.0	-12.77±0.12	-34.87±1.00	144.23±2.81	190.47±1.25	0.18±0.01	0.34±0.02
- neutral niosomes loaded with MM†								
45±2°C	5.0	4.2	-13.37±0.33	-27.03±0.52	154.57±1.10	391.43±4.72	0.21±0.01	0.46±0.01
RT (30±2°C)	5.0	4.2	-13.37±0.33	-35.90±0.59	154.57±1.10	225.67±4.32	0.21±0.01	0.49±0.01
4±2°C	5.0	4.4	-13.37±0.33	-27.73±0.60	154.57±1.10	222.77±1.25	0.21±0.01	0.49±0.01
Cationic niosomes								
- blank cationic niosomes	5.0	5.0	59.77±0.70	55.07±1.11	112.70±0.84	121.23±0.98	0.21±0.02	0.25±0.01
- cationic niosomes loaded with MM †								
45±2°C	4.8	4.0	66.73±0.45	52.70±1.46	116.43±1.53	130.90±1.57	0.19±0.01	0.20±0.02
RT (30±2°C)	4.8	4.5	66.73±0.45	49.00±1.18	116.43±1.53	153.20±0.78	0.19±0.01	0.39±0.01
4±2°C	4.8	4.6	66.73±0.45	52.70±1.46	116.43±1.53	181.93±2.40	0.19±0.01	0.48±0.05
Anionic niosomes								
- blank anionic niosomes	4.0	4.0	-57.73±2.19	-34.40±1.39	118.40±0.66	156.90±1.47	0.32±0.00	0.45±0.03
- anionic niosomes loaded with MM†								
45±2°C	4.0	3.5	-50.17±0.47	-39.10±0.89	148.17±2.14	173.83±2.20	0.31±0.02	0.40±0.04
RT (30±2°C)	4.0	3.6	-50.17±0.47	-31.30±1.18	148.17±2.14	165.30±2.05	0.31±0.02	0.39±0.02
4±2°C	4.0	3.8	-50.17±0.47	-44.63±0.19	148.17±2.14	239.05±2.47	0.31±0.02	0.64±0.05

†The loading concentrations of MM in neutral, cationic and anionic niosomes were 4.5, 11 and 0.1% w/w, respectively.

vesicular size than the loaded neutral niosomes. The incorporation of the charged molecule in niosomes as well as the negative charge of the loading substance appeared to affect the net charge of the vesicles and gave the reduced vesicular size. Also, this may be from the mechanism of the charged vesicular forming that spontaneously occurs in the hydration process. Again, the development of vesicular charge in the membranes before the vesicle formation may play the role in the rigidity and the rate of curvature of the bilayer membrane, resulting in the smaller vesicular size[75,216].

After 3 months at all storage temperatures, no physical change of all niosomal systems was observed. The pH values of neutral, cationic and anionic niosomes loaded with MM kept at 4±2, room temperature and 45±2°C for 3 months, which were about 4.3, 4.4 and 3.6, respectively, were lower than at initial (5.0, 4.8 and 4.0 for the loaded neutral, cationic and anionic niosomes, respectively). The lower pH may be from the hydrolysis of the acyl chain of the non-ionic surfactant containing in the niosomes. Brij72 is a non-ionic surfactant molecule composed of the polyoxyethylene as the polar group linked with the stearyl as the non-polar acyl group. In the niosomal formulation, Brij72 which is in contact with the aqueous media may be hydrolysed. MM can also be hydrolysed to give the acidic degradable compound (myristic acid)[56,220].

3.4.5 Chemical stability of MM loaded in the selected niosomes

Chemical stability of MM loaded in niosomal dispersions when kept at 4±2, room temperature and 45±2°C for 3 months was compared to the dry MM as shown in Fig. 37. After 3 months, the percentages remaining of MM in the dry form at 4±2, room temperature (30±2) and 45±2°C were 97.82, 96.56 and 91.39%, respectively.

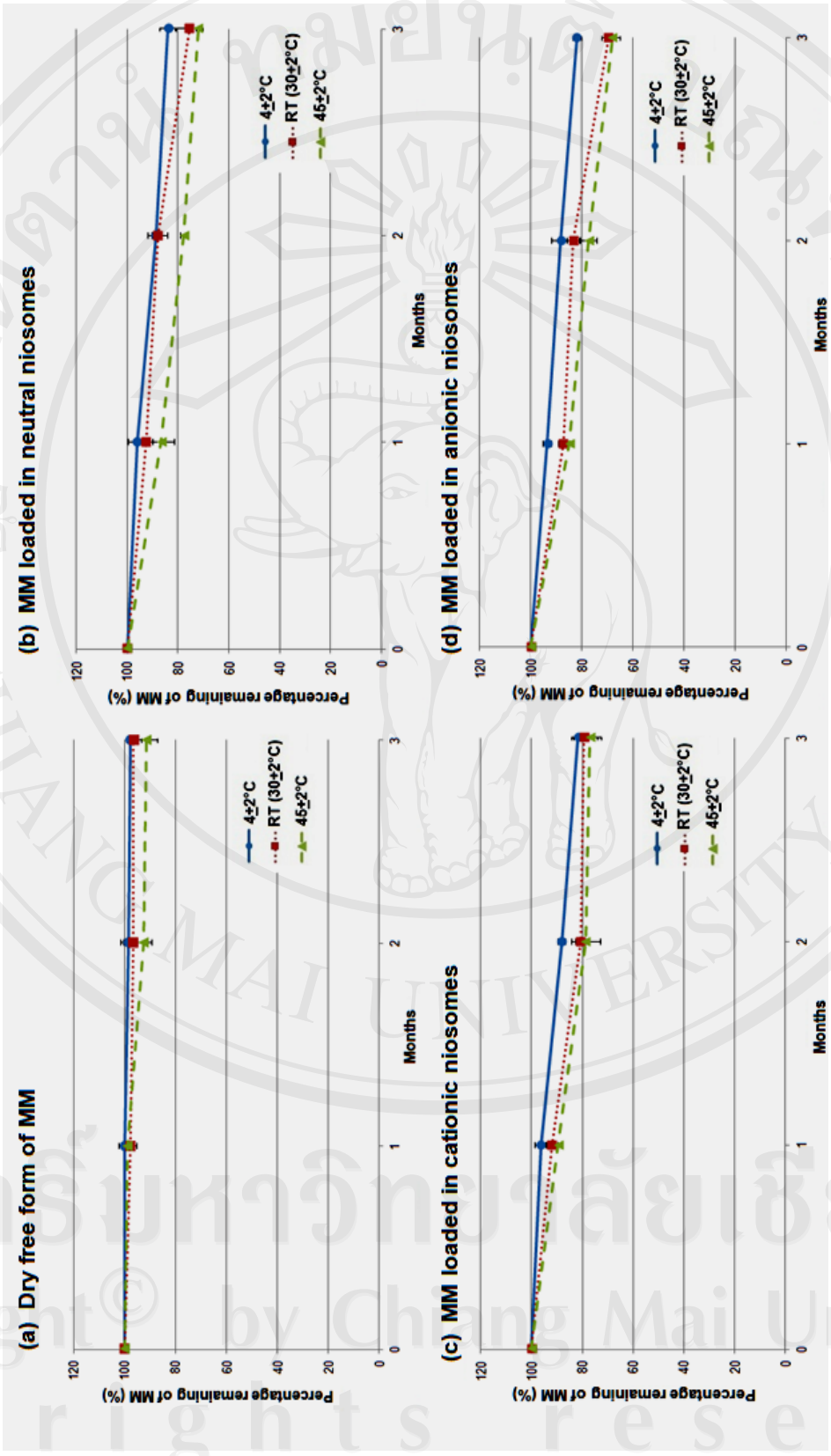


Figure 37 Percentage remaining of the dry free form of MM (a) and that loaded in neutral (b), cationic (c) and anionic (d) niosomes when kept at 4±2, room temperature (30±2) and 45±2°C for 3 months

The percentages remaining of MM loaded in neutral, cationic and anionic niosomes after 3 months at 4±2, room temperature and 45±2°C were 83.96, 75.59, 72.16; 81.68, 79.27, 76.97 and 82.06, 69.59, 68.05%, respectively. As the storage period and temperature increased, the remaining amount of MM decreased. The storage temperature at 4°C demonstrated higher stability of MM both in the dry free form and that entrapped in niosomes than at the other two storage high temperatures. The higher temperature could accelerate the degradation of MM. The chemical stability of the dry form MM was higher than that loaded in niosomes. Since MM loaded in niosomal dispersion may be hydrolyzed by water in the acidic environment (in the pH range of 3-5)[56]. For the effect of the charged niosomes on MM stability, MM loaded in cationic and neutral niosomes demonstrated higher stability than that loaded in anionic niosomes at room temperature and 45±2ºC. The low stability of MM loaded in anionic niosomes may be due to the effect of not only the storage temperature, but also the electrostatic interaction of the charged molecule and the negatively charged MM. As the negatively charged MM was loaded in niosomes, the order of tightly to loosely packed bilayers was expected to be cationic, neutral and anionic niosomes, respectively. MM loaded in the loosely packed vesicles may be liberated and eventually hydrolyzed to give the degradable compounds[221].

3.5 *In vitro* cell cytotoxicity, melanogenesis induction activity and transfollicular penetration assays of MM loaded in the selected niosomes

3.5.1 Cell cytotoxicity by SRB assay in human skin fibroblasts and B16F10 melanoma cells

For the cytotoxicity assay of the niosomal formulations in B16F10

melanoma cells, the treated sample concentrations and the exposure time to the cells of the blank neutral and cationic niosomes were investigated to obtain the appropriate conditions for the further study. The appropriate treated niosomal concentration and incubation time, which gave more than 80% viable cells, were at 0.20 mM and 24 h, respectively (Fig. 38).

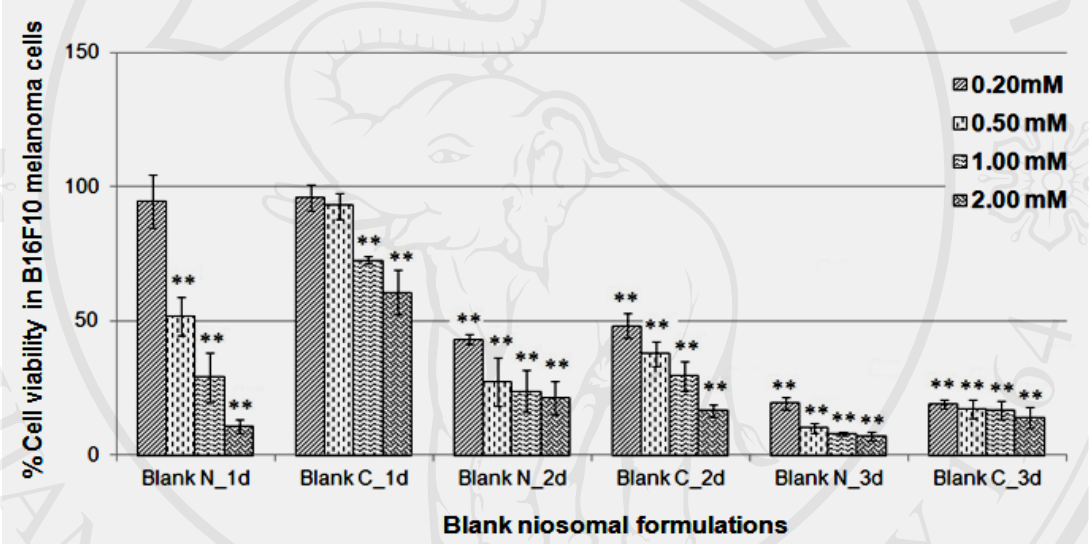


Figure 38 Percentage cell viability of B16F10 melanoma cells treated with the blank neutral (Blank N) and the blank cationic (Blank C) niosomes at the concentrations of 0.20, 0.50, 1.00 and 2.00 mM at 24- (_1d), 48- (_2d) and 72- (_3d) h incubation times. Data represented mean ± S.E. of three independent experiments. *Significant difference from the control (*$p < 0.05$,**$p < 0.01$)

Theophylline at 0.28 mM was not cytotoxic to both human skin fibroblasts and B16F10 melanoma cells (Fig. 39). Although theophylline slightly decreased cell viability of the fibroblasts (95.18±2.95% of the control), it did not suppress the cell proliferation of B16F10 melanoma cells (105.19±1.03% of the control). According to

the cytotoxic ratio of cell viability in normal and cancer cells of theophylline at 0.90±0.03, no toxic effect of theophylline to normal cells was indicated. However, the higher cell viability of tumor cells than the normal cells may be from the more tolerance of the tumor cells than the normal cells[222].

Cell viability of human skin fibroblasts and B16F10 melanoma cells treated with various blank niosomes and niosomes loaded with MM at 0.20 mM were presented in Fig. 39. Blank neutral niosomes and neutral niosomes loaded with MM demonstrated moderate cytotoxicity (56.64±3.19%, 47.34±2.13% in fibroblasts; 59.72±1.51%, 52.67±2.78% in B16F10 melanoma cells, respectively), while blank anionic niosomes and anionic niosomes loaded with MM indicated no cytotoxicity with more than 80% cell viability both in fibroblasts and B16F10 melanoma cells. For blank cationic niosomes and cationic niosomes loaded with MM, they exhibited moderate cytotoxic effects (73.81±2.86%, 73.20±3.49% in fibroblasts; 82.51±0.20%, 84.34±2.75% in B16F10 melanoma cells, respectively) to both cells with the suppression on fibroblast proliferation more than B16F10 melanoma cells. The free MM at 0.33, 13.60 and 34.80 μM treated fibroblasts and B16F10 melanoma cells showed high cell viability (more than 95%). Thus, the cytotoxic effect in both cells may be from niosomes, but not from MM. Cholesterol has been reported to inhibit cell proliferation with the IC_{50} value of 1746.95 μM, while DDAB promotes cell proliferation[162]. Also, the previous reports have investigated the toxicity of the non-ionic surfactants, such as Polysorbate80 and Cremophor EL, in rat thymocytes and lymphocytes. The cellular glutathione levels decreased after the exposure of the non-ionic surfactants, resulting in the increased hydrogen peroxide that eventually caused the oxidative stress and cell death[223-225]. The cytotoxic effect of Brij78 in the cultures

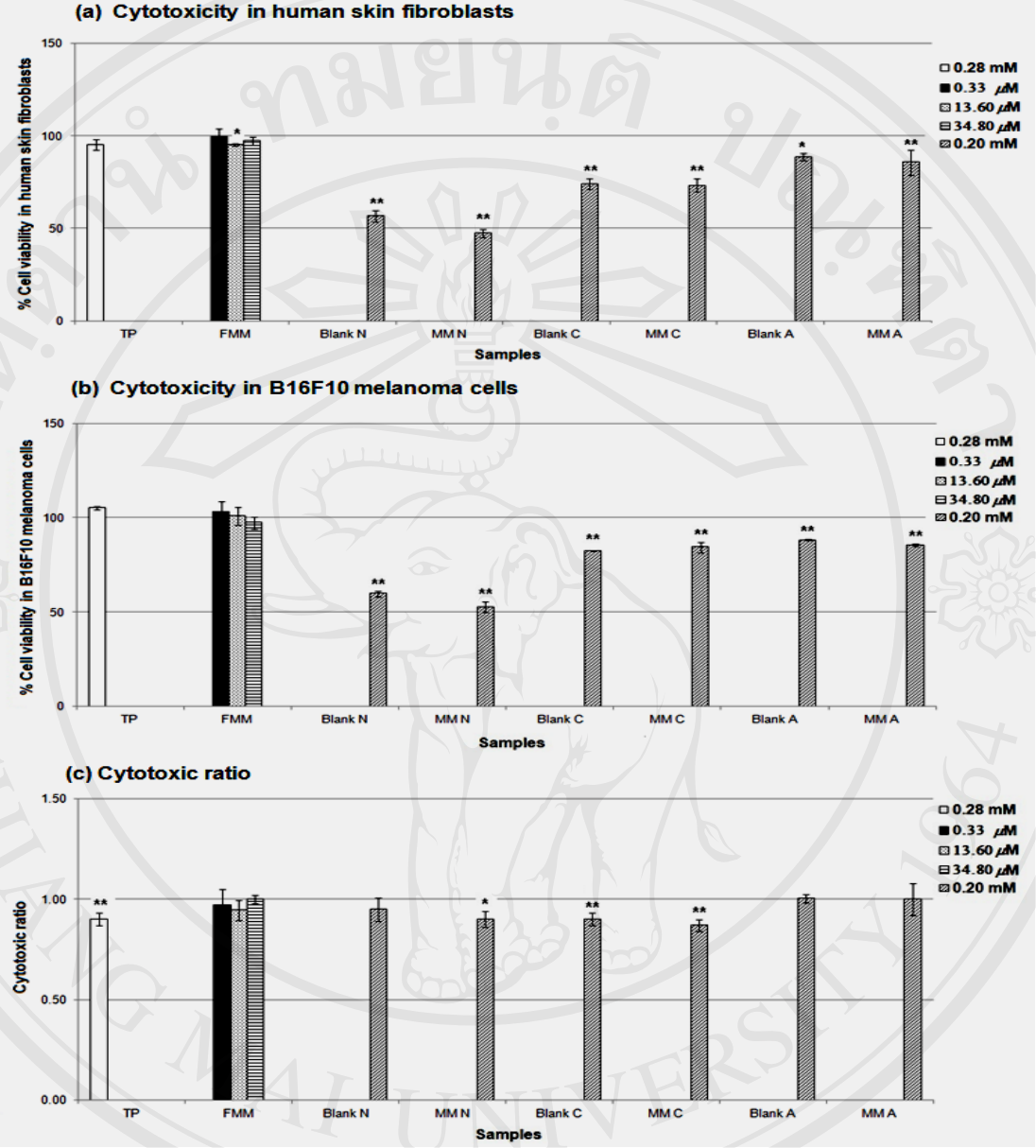


Figure 39 Cytotoxicity of theophylline (TP), free MM (FMM), blank neutral niosomes (Blank N) and neutral niosomes loaded with MM (MM N), blank cationic niosomes (Blank C) and cationic niosomes loaded with MM (MM C), blank anionic niosomes (Blank A) and anionic niosomes loaded with MM (MM A) in human skin fibroblasts (a), B16F10 melanoma cells (b) and cytotoxic ratio (c) of cell viability in normal and cancer cells. Data represented mean $\pm$ S.E. of three independent experiments. *Significant difference from the control (*$p < 0.05$,**$p < 0.01$)

of rabbit and human corneal epithelial cells demonstrated the reduced cytotoxicity when bound with fetal bovine serum[226]. When DP was incorporated in the docetaxel liposomes, the drug exhibited lower toxicity than the free drug in HT-29 colon adrenocarcinma and Igrov-1 ovarian carcinoma cells[227]. According to the previous assessment of skin irritation of cosmetics, the cut-off value of cell viability determined by MTT cell viability assay at 50% was indicated the cytotoxicity of sample[178]. In this study, the three developed blank niosomes showed cell viability both in fibroblasts and B16F10 melanoma cells higher than 50%, while cationic and anionic niosomes loaded with MM also demonstrated cell viability higher than 50%. Different niosomal compositions and concentrations affected the cytotoxicity. The observed moderate cytotoxicity of neutral niosomes may be from the Brij72 and cholesterol contents since the cytotoxicity of these two compounds have been reported[162, 223-225]. The lowest cell viability of blank neutral niosomes and neutral niosomes loaded with MM observed in both fibroblasts and B16F10 melanoma cells may be due to the higher concentration of Brij72 and cholesterol than in the blank cationic, blank anionic and those loaded with MM. The lower cell viability of both cationic niosomes loaded and not loaded with MM than anionic niosomes loaded and not loaded with MM observed in both cells may be due to the enhanced intracellular penetration of the compound by the positively charged molecule containing in cationic niosomes[228-230]. The cytotoxic ratios of cell viability in normal and cancer cells of blank neutral, blank cationic, blank anionic and these niosomes loaded with the MM were 0.95$\pm$0.06, 0.90$\pm$0.03, 1.00$\pm$0.02, 0.90$\pm$0.04, 0.87$\pm$0.03 and 1.00$\pm$0.08, respectively, while the ratios of the free MM at 0.33, 13.60 and 34.80 μM were 0.97$\pm$0.08, 0.95$\pm$0.05 and 1.00$\pm$0.02, respectively. All niosomal systems indicated

similar cell viability of both normal and cancer cells, except the cationic niosomes loaded with MM which gave lower viable cells of fibroblasts than B16F10 melanoma cells owing to the less tolerance of the normal cells than the tumor cells[222]. Most of the developed blank and MM loaded niosomes appeared not to be toxic to the normal cells.

3.5.2 Melanin content measurement

For the melanogenesis induction assay, the different concentrations of the test samples were obtained from the preliminary cytotoxicity, melanogenesis evaluation and the maximum loading of MM in niosomes. The preliminary cytotoxicity assay of the blank niosomes was investigated to obtain the appropriate conditions for the melanogenesis assay, including the incubation time and niosomal concentration. Incubation time of niosomes could also affect the melanogenesis. The 72-h incubation time of theophylline at 10 μg/ml (0.06 mM) demonstrated the melanogenesis stimulation effect in our previous study. But, the melanin induction at 24-h incubation could not be detected. However, when the concentration of theophylline was increased to 50 μg/ml (0.28 mM), the melanin induction at this incubation period could be observed. The concentrations of the free MM at 0.33 (0.08 μg/ml), 13.60 (3.30 μg/ml) and 34.80 (8.43 μg/ml) μM were obtained from the maximum loading of MM in anionic, neutral and cationic niosomes, respectively, while the niosomal concentration at 0.20 mM was from our preliminary cytotoxicity. Although different concentrations of all test samples were used, the data were finally normalized with the total protein content of each treatment for the comparison.

Theophylline, a melanogenesis stimulator, was used as a positive control. Pigment regulation mechanism of theophylline is mediated through cAMP pathway as

well as the increasing of the gamma-glutamyl transpeptidase- and tyrosinase-reactive cells, resulting in the induction of melanin production with the increased level of tyrosinase activity[206-207]. Theophylline at 0.28 mM demonstrated the melanin induction at 1.24 folds of the control, whereas the free MM at 0.33, 13.60 and 34.80 μM showed the melanin induction at 1.06, 1.19 and 1.42 folds of the control, respectively (Fig. 40). The highest melanin induction of the free MM was observed at 34.80 μM. The high melanin induction potency of MM may be due to the lipophilicity of MM that may be efficiently taken up by the cells. Thus, MM stimulated the melanin production more than theophylline. The blank neutral and cationic niosomes, except the blank anionic niosomes, also significantly stimulated the melanin production higher than the control, owing to the high content of cholesterol in the neutral niosomes and the positively charged molecule in the cationic niosomes. In fact, the involvement of cholesterol in the pigmentation biosynthesis and tyrosinase expression through the extracellular signal regulated kinase (ERK) pathway has been reported[231]. Also, the positively charged molecules, such as the cationic cell-penetrating peptides (CPP)[228], cationic niosomes and liposomes[229-230], can enhance the intracellular delivery of various compounds, such as antisense oligonucleotides and plasmid DNA. Neutral, cationic and anionic niosomes loaded with MM significantly induced melanin production higher than their corresponding blank formulations of 1.10, 1.16 and 1.24 folds, respectively (Fig. 40). Therefore, this melanin biosynthesis stimulation may be from the synergistic effects of MM and the niosomal compositions, not only the stimulation of melanin production, but also the penetration enhancement of MM as well.

3.5.3 Tyrosinase activity measurement

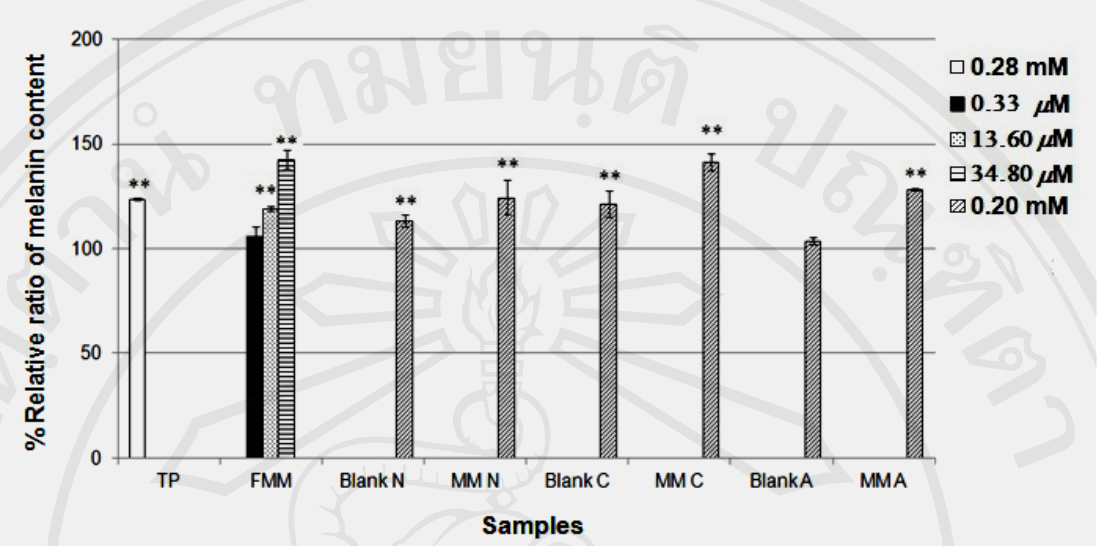


Figure 40 Percentage relative ratio of melanin content of B16F10 melanoma cells treated with theophylline (TP), free MM (FMM), blank neutral niosomes (Blank N) and neutral niosomes loaded with MM (MM N), blank cationic niosomes (Blank C) and cationic niosomes loaded with MM (MM C), blank anionic niosomes (Blank A) and anionic niosomes loaded with MM (MM A). Data represented mean ± S.E. of three independent experiments. *Significant difference from the control (*$p < 0.05$,**$p < 0.01$)

Tyrosinase activity of theophylline at 0.28 mM was 1.07 folds of the control. The tyrosinase activity values of the free MM at 0.33, 13.60 and 34.80 μM were 1.04, 1.15 and 1.48 folds of the control, respectively (Fig. 41). The tyrosinase activity of the free MM appeared to be concentration-dependent and was related to the melanin induction activity with the correlation coefficient (R^2) of 0.818 (p<0.01). This result agreed with our previous study that melanogenesis enhancement of MM showed the positive correlation of melanin stimulation and tyrosinase activity.

The neutral niosomes loaded and not loaded with MM, cationic niosomes

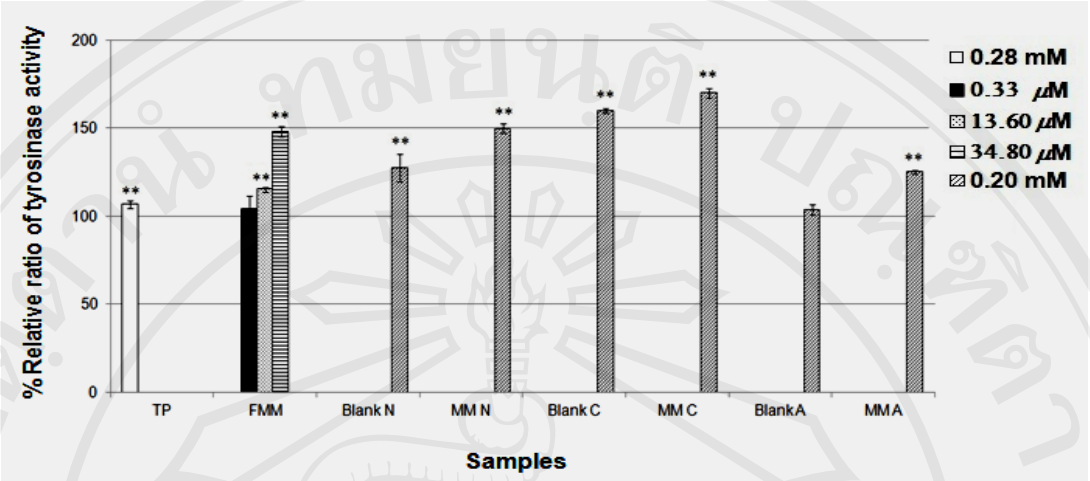


Figure 41 Percentage relative ratio of tyrosinase activity of B16F10 melanoma cells treated with theophylline (TP), free MM (FMM), blank neutral niosomes (Blank N) and neutral niosomes loaded with MM (MM N), blank cationic niosomes (Blank C) and cationic niosomes loaded with MM (MM C), blank anionic niosomes (Blank A) and anionic niosomes loaded with MM (MM A). Data represented mean ± S.E. of three independent experiments. *Significant difference from the control cells (*$p < 0.05$,**$p < 0.01$).

loaded and not loaded with MM, anionic niosomes loaded and not loaded with MM showed the tyrosinase activity of 1.50, 1.27, 1.70, 1.60, 1.25 and 1.04 times higher than the control, respectively. Also, MM loaded in neutral, cationic and anionic niosomes demonstrated the tyrosinase activity of 1.30, 1.15 and 1.21 times, respectively, higher than the free MM at the same concentration (Fig. 41). Similarly, the increased tyrosinase activity of MM loaded in niosomes correlated with the melanin induction activity. This correlation may be from the synergistic effects of MM and the niosome compositions.

3.5.4 Tyrosinase-related protein-2 activity measurement

The melanogenesis induction of methyl palmitate has been reported to involve in the TRP-2 expression[7]. In this study, the TRP-2 activity of MM and MM loaded in niosomes was evaluated based on the consumption of dopachrome. The higher TRP-2 activity than the control demonstrated the lower relative ratio than the control (100%). The TRP-2 activity of theophylline at 0.28 mM was 1.07 folds of the control. The free MM at 0.33, 13.60 and 34.80 μM exhibited 1.07, 1.07 and 1.20 times higher TRP-2 activity than the control, respectively (Fig. 42). These results were in agreement with the previous report on the induction of TRP-2 expression in melanin biosynthesis stimulation[7].

The blank neutral niosomes, blank cationic niosomes, neutral and cationic niosomes loaded with MM exhibited the TRP-2 activity of 1.76, 1.68, 1.78 and 1.47 times, respectively, lower than the control, while the blank anionic niosomes and anionic niosomes loaded with MM showed similar TRP-2 activity to the control (Fig. 42). MM loaded in all niosomal systems significantly demonstrated lower TRP-2 activity than the free MM at the same concentration (0.33, 13.60 and 34.80 μM). The loaded and not loaded niosomes with MM have different melanogenesis stimulation action from the free MM. Melanin biosynthesis in the pigment producing cells involves various steps. The pigment stimulation compounds can interact in many steps of the process. Dopachrome, the pigment intermediate, is first oxidized to DHICA by TRP-2. Also, dopachrome could be oxidized by the certain divalent metal cations as well as spontaneously formed DHI (5,6-dihydroxyindole) in the absence of TRP-2 enzyme[168,232]. Although the pigment regulation of the saturated fatty acid methyl ester has been reported to mediate through the expression of TRP-2, the loaded and not loaded niosomes with MM composing of various compounds,

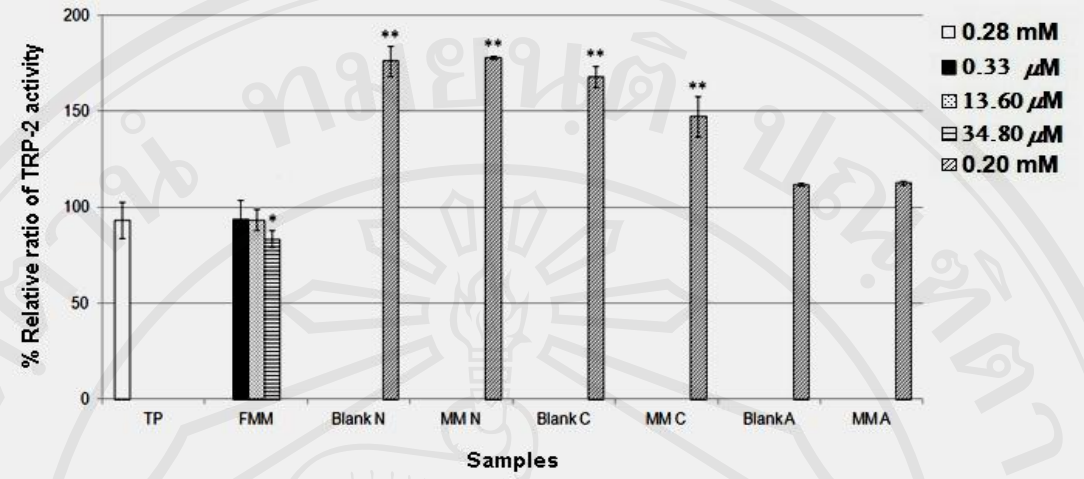


Figure 42 Percentage relative ratio of TRP-2 activity of B16F10 melanoma cells treated with theophylline (TP), free MM (FMM), blank neutral niosomes (Blank N) and neutral niosomes loaded with MM (MM N), blank cationic niosomes (Blank C) and cationic niosomes loaded with MM (MM C), blank anionic niosomes (Blank A) and anionic niosomes loaded with MM (MM A). TRP-2 activity based on the comsumption of the dopachrome was calculated as the percentage of the relative ratio of the TRP-2 activity according to the following equation: % relative ratio of TRP-2 activity = (Dt/Dc) × 100, where Dt was the TRP-2 activity of sample divided by total protein content of sample and Dc was the TRP-2 activity of control divided by total protein content of control. Data represented mean ± S.E. of three independent experiments. *Significant difference from the control cells (*$p < 0.05$,**$p < 0.01$).

including the non-ionic surfactant, cholesterol and the charged lipid, may increase the melanin production through other stimulation pathways, such as the expression of tyrosinase and the upregulation of MITF, more than the TRP-2 activity[7,231].

3.5.5 Transfollicular penetration

The *in vitro* skin permeation is used to predict the percutaneous penetration

in humans. The morphology, function as well as the penetration of topically applied substances of the porcine skin have been reported to be close to the human skin[233-234]. For the porcine skin preparation, the scalpel was used to remove the excess subcutaneous fat by the pinching method, which did not affect the hair follicles during the procedure[235-236]. Recently, hair follicles have been interested as the important target for drug delivery due to their surrounding is closed to the network of blood capillaries and dendritic cells[151]. Different approaches, such as skin sandwich model, selective hair follicular blocking and differential stripping, have been used to investigate the follicular penetration[149,152,154,156-158,237]. In this study, the selective hair follicular blocking and differential stripping have been employed on the porcine skin. Two skin systems, including opened and blocked hair follicle skin, were prepared to evaluate the follicular penetration of MM from solution and various niosomal formulations. The topical penetration of MM through the opened hair follicle skin mounted on vertical Franz diffusion cells was mediated through both the transepidermal and follicular routes, whereas the penetration through the blocked hair follicle skin was only through the transepidermal route. The difference flux between the opened hair follicle skin and blocked hair follicle skin was determined as the transfollicular penetration flux[236].

The cumulative amounts of MM in skin and receiver compartment at 1, 2, 4 and 6 h were shown in Fig. 43. The cumulative amounts, fluxes and follicular penetration per one hair follicle by the follicular closing technique using vertical Franz diffusion cells at 6 h of MM from solution and various niosomal formulations were in Table 15. The cumulative amounts of MM from all formulations through skin increased with times. In the skin after 6 h, the cumulative amounts of MM loaded in

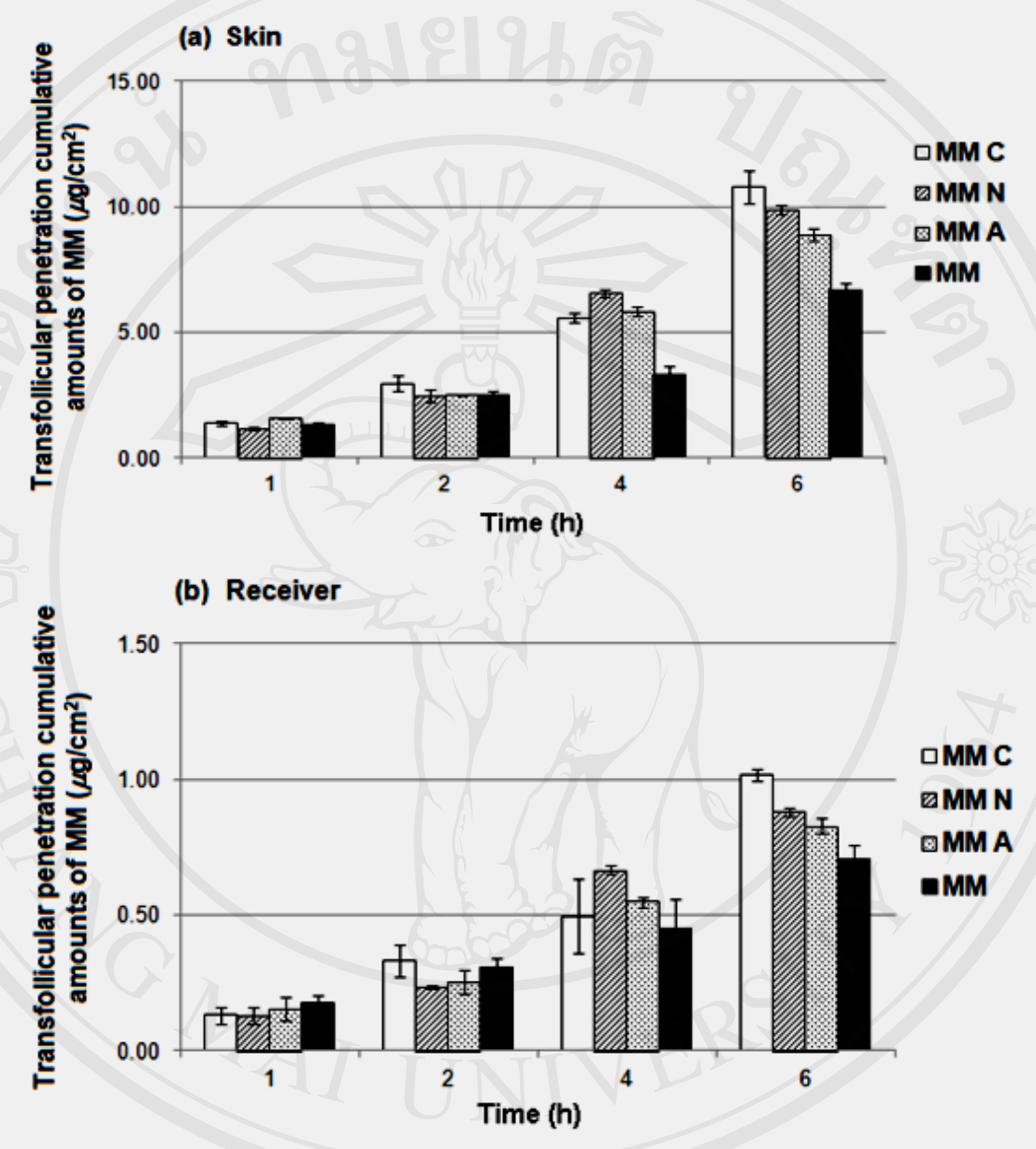


Figure 43 Cumulative amounts of MM (μg/cm^2) from solution (MM), cationic niosomes (MM C), neutral niosomes (MM N) and anionic niosomes (MM A) in skin (a) and the receiver (b) by follicular closing technique using vertical Franz diffusion cells at 0, 1, 2, 4 and 6 h

Table 15 The cumulative amounts ($\mu g/cm^2$), fluxes ($\mu g/cm^2/h$) and follicular penetration per one hair follicle (μg/one hair follicle) by follicular closing technique using vertical Franz diffusion cells at 6 h of MM from solution and various niosomal formulations

Formulation	Skin			Receiver		
	Cumulative amounts ($\mu g/cm^2$)	Fluxes ($\mu g/cm^2/h$)	Follicular penetration per one hair follicle (μg/one hair follicle)	Cumulative amounts ($\mu g/cm^2$)	Fluxes ($\mu g/cm^2/h$)	Follicular penetration per one hair follicle (μg/one hair follicle)
MM loaded in cationic niosomes (MM C)	10.7720	1.7953	0.1393	1.0149	0.1692	0.01312
MM loaded in neutral niosomes (MM N)	9.8930	1.6488	0.1248	0.8802	0.1467	0.0111
MM loaded in anionic niosomes (MM A)	8.8795	1.4799	0.1003	0.8281	0.1380	0.0094
MM in 70% (v/v) propylene glycol (MM)	6.6860	1.1143	0.0729	0.7077	0.1180	0.0077

cationic (10.7720 μg/cm^2), neutral (9.8930 μg/cm^2) and anionic (8.8795 μg/cm^2) niosomes were higher amounts than that from solution (6.6860 μg/cm^2) of about 1.61, 1.48 and 1.33 times, respectively. The similar cumulative amounts of MM were also observed in the receiver compartment. At 6 h, MM loaded in cationic (1.0149 μg/cm^2), neutral (0.8802 μg/cm^2) and anionic niosomes (0.8281 μg/cm^2) showed higher cumulative amounts than that in solution (0.7077 μg/cm^2) of 1.43, 1.24 and 1.17 times, respectively, in the receiver chamber. Although propylene glycol incorporated in the MM solution was expected to improve the follicular penetration, the cumulative amounts of MM in this solution were less than MM loaded in niosomes[53]. These results suggested that niosomes might enhance the follicular penetration of MM[92,95,112,236,238]. The charge of niosomes also affected follicular penetration. The order of the highest to the lowest cumulative amounts of MM was observed in the following loaded niosome: in cationic, neutral and anionic niosomes. The influence of the surface charge on skin permeation have been reported that the cationic compound increases skin permeability of the treated drug[239]. Thus, the skin permeation of MM may be improved when loaded in the cationic charged niosomes. The results of the enhanced follicular penetration of MM when loaded in different charged niosomes were in the agreement with the previous report[239].

The penetration depth depends on the particle size as well as the surface charge of the penetrating substance[136, 240]. Since the porcine skin has the pH of 6-7, the penetrant may face with the acidic environment from the skin itself and the secreted sebum consisting of high contents of free fatty acids[240-241]. The 400 nm penetrating vesicles with the amphoteric and the cationic charge have been exhibited in the full hair follicle length of approximately 70%[240]. The particle size of MM

loaded in various niosomal systems were in the range of 112-155 nm. This expected that the penetration of MM loaded in vesicles was permeated into the infundibulum of the hair follicles composing of melanocytes, the target for canities treatment.

3.5.6 Selection of MM loaded in the selected niosomes in order to further incorporate in the hair lotion formulation

MM was loaded in neutral, cationic and anionic niosomes consisting of Brij72, cholesterol with and without the charged lipid (DDAB or DP). The *in vitro* cytotoxicity of the various charged niosomes loaded with MM was investigated as well as the melanin induction activity and transfollicular penetration using vertical Franz diffusion cells were also examined in comparing to the free MM at the same concentration. MM loaded in cationic niosomes composing of Brij72/cholesterol/ DDAB at 7:3:0.65 molar ratio that showed the melanin induction activity, transfollicular penetration and good physicochemical properties higher than the free MM and that loaded in neutral and anionic niosomes were selected to further incorporate in the hair lotion formulation.

3.6 Development of the hair lotion bases

3.6.1 Interaction study of the non-ionic surfactants and the selected niosomes loaded with MM

Surfactants are widely used as detergents, wetting agents, emulsifiers, foaming agents and dispersants in pharmaceutical and cosmetic formulations. Among various types of surfactants, the non-ionic surfactants are most commonly employed due to their lower toxicity in comparing to anionic and cationic surfactants[242-243]. However, the erythrocyte hemolysis induced by non-ionic surfactants has been

reported[244-245]. Since liposomes and niosomes are the closed bilayer vesicles formed from the non-ionic amphiphiles in aqueous media, the lipid bilayer membranes of niosomes are similar to the erythrocyte membranes. The surfactant can affect the lipid bilayer membrane by changing the closed bilayer structure and lead to the rupture of the membrane. The rupture of the vesicular membrane is believed to occur from the partitioning of surfactant into the vesicular membrane, followed by the formation of surfactant-lipid mixed micelles and the rupture of the membrane, resulting in the change of the vesicle physical characteristics[246-247].

In this study, the non-ionic surfactants with high hydrophilic-lipophilic balance (HLB) value were interacted with the membrane of cationic niosomes loaded with MM. The vesicular membrane structures of niosomes were changed. Turbidity and vesicular size measurement were observed. The percentage changes of turbidity and vesicular size of the non-ionic surfactant mixture, including HC-60, HC-100, TS-10V, PEN-4620, GT-20IS, Decaglyn 1-IS, Sunsoft Q-192Y, L-1695 and Tween20, and cationic niosomes loaded with MM were shown in Fig. 44 and 45, respectively.

The turbidity of the mixture of distilled water and cationic niosomes loaded with MM (control) was 0.0821$\pm$0.0025, while that of most mixtures (non-ionic surfactants and niosomes) indicated the increased turbidity. Only L-1695 decreased turbidity of 0.24-fold of the control. The turbidity changes of less than $\pm$15% were observed in the mixture of non-ionic surfactants, including HC-60 (5.06$\pm$1.48%), HC-100 (10.45$\pm$0.21%), PEN-4620 (5.41$\pm$2.18%), TS-10V (12.59$\pm$2.74%) and Tween20 (11.41$\pm$0.07%), and cationic niosomes loaded with MM. The vesicular size of cationic niosomes loaded with MM (the control) was 184.68$\pm$2.53 nm, whereas that of most mixture of non-ionic surfactant and niosomes decreased. The increased

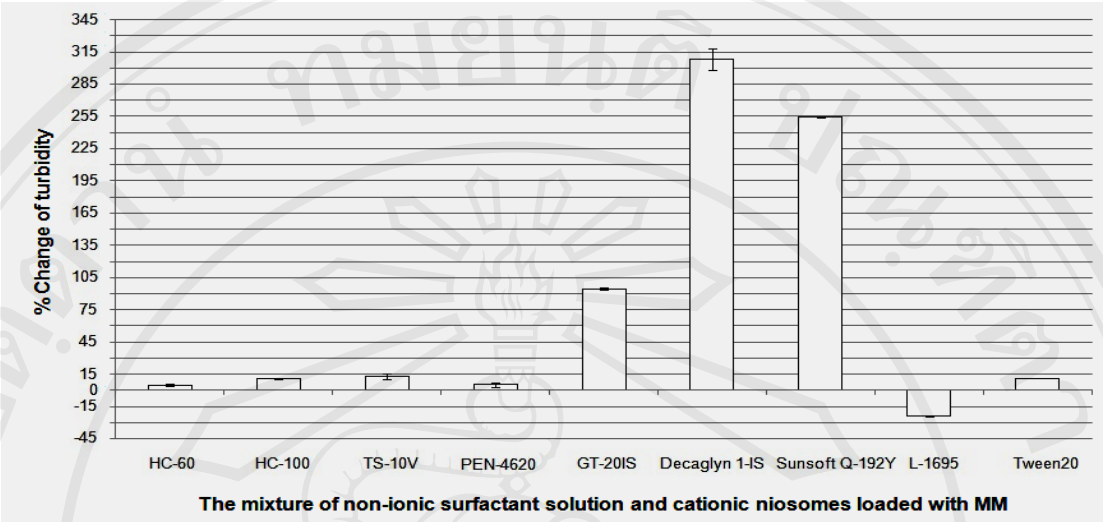


Figure 44 Percentage changes of turbidity obtained from the mixture of non-ionic surfactants, including HC-60, HC-100, TS-10V, PEN-4620, GT-20IS, Decaglyn 1-IS, Sunsoft Q-192Y, L-1695 and Tween20, and cationic niosomes loaded with MM

vesicular size of the mixture of Decaglyn 1-IS, HC-60 and Sunsoft Q-192Y and cationic niosomes loaded with MM were 0.26, 0.03 and 1.37 times, respectively, larger than control. The vesicular size changes of less than $\pm$15% were found in the mixture of non-ionic surfactants, including GT-20IS (7.56$\pm$0.27%), HC-60 (3.38$\pm$0.26%), HC-100 (-1.67$\pm$5.47%), PEN-4620 (-11.26$\pm$5.76%) and Tween20 (-14.46$\pm$4.12%), and cationic niosomes loaded with MM. The cut-off percentage change in physical characteristics of vesicular membrane observed from the interaction of non-ionic surfactant and vesicles was set at $\pm$15% for the appropriate selection of non-ionic surfactant to incorporate in the hair lotion base formulations.

3.6.2 Selection of the non-ionic surfactants for hair lotion base formulations

The non-ionic surfactants that exhibited the change in membrane structure of niosomal vesicles of less than $\pm$15% determined by turbidity and vesicular size

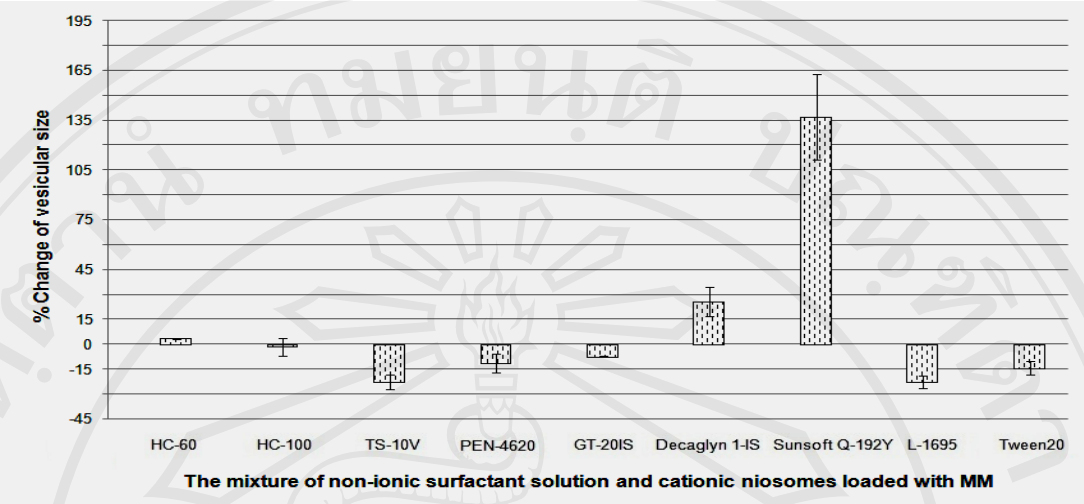


Figure 45 Percentage changes of vesicular size of the mixture of non-ionic surfactant, including HC-60, HC-100, TS-10V, PEN-4620, GT-20IS, Decaglyn 1-IS, Sunsoft Q-192Y, L-1695 and Tween20, and cationic niosomes loaded with MM

measurement, including HC-60, HC-100, PEN-4620 and Tween20, were selected to incorporate in the hair lotion base formulations.

3.6.3 Physical characteristics and thermodynamic stability of the hair lotion base formulations

The understanding of the membrane rupture by surfactant is not fully understood, although the possible mechanism has been reported[244,247]. This phenomenon is depended on the surfactant type, initial surfactant concentration and experimental conditions, such as ionic strengths, pH and temperature[248-249]. Various factors can affect the vesicular membrane rupture. The heating and cooling method at 45 and 4°C is an accelerated condition generally used for thermodynamic stability evaluation[250-251]. Tables 16-17 demonstrated the physical characteristics of the hair lotion base formulations at initial and after 6 cycles of heating and cooling. Only stable hair lotion base formulations with no phase separation were investigated for

Table 16 Physical characteristics (appearance, odor, specific gravity and viscosity) of the hair lotion base formulations at initial and after 6 cycles of heating and cooling (45/4°C)

Formula No.	Initial					After 6 cycles of heating and cooling method			
	Appearance	Odor	Specific gravity (g/ml)	Viscosity* (cP)	Thermodynamic stability**	Appearance	Odor	Specific gravity (g/ml)	Viscosity* (cP)
1	turbid lotion	pungent odor	1.168	179.0	✓	turbid lotion	pungent odor	1.046	43.0
2	turbid lotion	pungent odor	0.9432	3270.0	✓	turbid lotion	pungent odor	1.798	1197.0
3	turbid lotion	pungent odor	1.094	16.1	×	-	-	-	-
4	turbid lotion	pungent odor	1.022	81.0	✓	turbid lotion	pungent odor	1.038	25.9
5	turbid lotion	pungent odor	1.356	313.0	✓	turbid lotion	pungent odor	1.056	95.0
6	turbid lotion	pungent odor	1.414	294.0	×	-	-	-	-
7	turbid lotion	pungent odor	1.158	196.0	✓	turbid lotion	pungent odor	0.994	83.0
8	turbid lotion	pungent odor	1.058	304.0	✓	turbid lotion	pungent odor	1.006	126.0
9	turbid lotion	pungent odor	1.036	64.3	×	-	-	-	-
10	turbid lotion	no odor	1.100	89.0	✓	turbid lotion	no odor	1.0600	28.8
11	turbid lotion	no odor	1.286	291.0	✓	turbid lotion	no odor	1.202	115.3
12	turbid lotion	no odor	1.022	245.0	✓	turbid lotion	no odor	1.314	108.0
13	turbid lotion	no odor	1.132	532.0	×	-	-	-	-
14	turbid lotion	no odor	0.9381	5.0	×	-	-	-	-
15	turbid lotion	no odor	0.9563	7.9	×	-	-	-	-

**Note ***Myr VR 3000 viscometer, spindle TL 5 and 6, 30-200 rpm, 25±2°C; **Visually observed thermodynamic stability after 6 cycles of heating and cooling method: ✓ no phase separation, × phase separation

Table 17 Physical characteristics (pH, Z-zeta potential, particle diameter and polydispersity index) of the hair lotion base formulations at initial and after 6 cycles of heating and cooling (45/4°C)

Formula No.	Initial				Thermodynamic stability**	After 6 cycles of heating and cooling method			
	pH	Z-zeta potential (mV)	Particle diameter (nm)	Polydispersity index		pH	Z-zeta potential (mV)	Particle diameter (nm)	Polydispersity index
1	6.0	-52.60±0.32	513.10±3.67	1.00±0.00	✓	5.5	-53.27±1.51	419.07±4.82	1.00±0.00
2	6.0	-30.80±0.50	825.00±3.64	0.67±0.00	✓	6.0	-51.7±0.65	1415.67±2.33	0.53±0.00
3	5.0	12.10±0.28	174.63±1.02	0.43±0.02	×	-	-	-	-
4	6.0	-58.80±0.65	360.30±3.32	1.00±0.00	✓	7.0	-59.93±0.79	883.73±3.91	0.73±0.00
5	6.0	-35.5±1.14	593.87±8.64	1.00±0.00	✓	6.0	-55.07±0.53	1276.67±9.56	0.80±0.05
6	6.0	7.59±0.51	198.30±0.79	0.48±0.01	×	-	-	-	-
7	6.0	-23.30±1.15	382.73±1.48	1.00±0.00	✓	6.0	-35.13±0.52	735.27±4.19	0.71±0.03
8	6.0	-13.90±1.09	254.20±5.86	1.00±0.00	✓	6.0	-37.10±1.20	312.20±2.19	1.00±0.00
9	5.0	5.21±0.10	205.57±2.26	0.56±0.03	×	-	-	-	-
10	6.0	-23.6±2.20	421.20±3.48	0.66±0.01	✓	5.5	-62.43±0.72	396.73±1.17	0.58±0.02
11	6.0	-26.3±2.02	493.40±4.49	0.64±0.01	✓	5.5	-58.57±0.82	554.33±1.93	0.59±0.02
12	5.0	3.84±0.15	212.13±0.55	0.43±0.03	✓	4.0	7.21±0.24	237.20±0.74	0.51±0.03
13	5.0	7.63±0.21	557.23±4.04	0.92±0.08	×	-	-	-	-
14	6.2	-13.6±0.58	493.40±4.49	0.64±0.01	×	-	-	-	-
15	6.2	-19.40±0.22	418.67±2.78	0.99±0.00	×	-	-	-	-

physical characteristics after thermodynamic stability study. Six of the total of 15 formulations were thermodynamic unstable with phase separation after 3-4 cycles of heating and cooling, whereas the rest of 9 formulations exhibited the stable formulations. The thermodynamic stable hair lotion base formulations may be from the appropriate proportion of the oil concentration, non-ionic surfactant concentration and water phase compositions[250].

Nine thermodynamic stable formulations were composed of different non-ionic surfactant concentration and the rheology modifier (CMC and HPMC). The addition of HPMC with or in place of CMC turned the zeta potential of the formulation to be more positive charge as observed in Formulas No. 2, 5, 8, 11 and 12. The zeta potential of most stable formulations, including Formulas No. 1, 2, 4, 5, 7, 8, 10 and 11, indicated the negative charge ranged from -62.43$\pm$0.72 to -35.13$\pm$0.52 mV, while Formula No. 12 showed the positive charge with the zeta potential of 7.21$\pm$0.24 mV. Also, the effects of the addition of HPMC on particle diameter and polydispersity index were observed. The particle diameter and polydispersity index of the formulations containing only HPMC (Formulas No. 3, 6, 9 and 12) were less than those consisted of CMC and the mixture of CMC and HPMC. This observation may be from the molecular weight of HPMC (22 kDa), which is less than CMC (90 kDa) of about 4 times, resulting in the tightly orientated structure of HPMC than CMC in the formulation[252].

3.6.4 Selection of the hair lotion base formulation to incorporate cationic niosomes loaded with MM

Fifteen hair lotion base formulations were prepared and investigated for physical characteristics by heating and cooling. Only 9 hair lotion base formulations

exhibited good physical stability with different specific gravity, viscosity, charge and particle size. The appropriate charge of the hair lotion base formulation should be positive because the positively charged niosomes loaded with MM was incorporated in this base. Thus, Formula No. 12 with zeta potential of 7.21±0.24 mV and particle size of 237.20±0.74 nm was selected for the further study.

3.7 Development of the hair lotion containing MM loaded and not loaded in cationic niosomes

3.7.1 Physical characteristics of hair lotion containing MM loaded and not loaded in cationic niosomes

The select hair lotion base formulation (Formula No. 12) was consisted of the non-ionic surfactant HC-60 used as the wetting agent, the oil phase composed jojoba oil and vitamin E, the water phase composed of trimethyl glycine, panthenol, glycerol, propylene glycol and water. Liquid germall plus and HPMC were used as the preservative and rheology modifier, respectively. All prepared hair lotions, including hair lotions containing MM loaded and not loaded in cationic niosomes and hair lotion base, were colorless turbid lotions. The physical characteristics of three formulations were shown in Table 18. All hair lotion formulations indicated pH in the range of 4.5-5.0, which were similar to the pH of the scalp and skin and appropriate to be used on the scalp and skin[253]. The zeta potential, particle size and polydispersity index of cationic niosomes loaded with MM were 33.60±1.80 mV, 143.50±0.12 nm and 0.35±0.00, respectively, whereas these niosomes that incorporated in hair lotion demonstrated the decreased zeta potential (4.66±0.59 mV) and the increased particle size (189.30±0.31 nm) and polydispersity index

Table 18 Physical characteristics of hair lotions containing MM loaded and not loaded in cationic niosomes and the hair lotion base

Formulation	Appearance	Odor	Specific gravity (g/ml)	Viscosity* (cP)	pH	Z-zeta potential (mV)	Particle diameter (nm)	Polydispersity index
Hair lotion containing MM loaded in cationic niosomes	turbid lotion	no odor	1.302	143.0	4.5	4.66±0.59	189.30±0.31	0.50±0.01
Hair lotion containing MM not loaded in cationic niosomes	turbid lotion	no odor	1.254	126.0	4.5	-15.60±0.55	219.40±2.02	0.32±0.02
Hair lotion base	turbid lotion	no odor	1.048	108.0	5	-16.83±0.38	93.06±0.19	0.16±0.01

Note *****Myr VR 3000 viscometer, spindle TL 6, 30-200 rpm, 25±2°C

(0.50±0.01). The changed physical characteristics of the hair lotion composing of MM loaded in cationic niosomes may be from the interaction between the niosomes and the hair lotion compositions. Since hair lotion base exhibited negative charge, owing to the negative charge of HPMC (the zeta potential of 0.1% suspension of HPMC in water was -13.77±0.38 mV), the addition of the positively charged niosomes loaded with MM would turn the charge of the formulation to be more positive than the base formulation. Although the zeta potential of all hair lotion formulations were in the range of the unstable values (within the range of ±30 mV)[79], neither phase separation nor the unstable aggregation was observed during the previous thermodynamic stability evaluation. However, the stable hair lotion formulations may be from the presence of the swelling agent, HPMC. HPMC is a rheology modifier that can stabilize other molecules from its long polymeric chain as well as suspend other molecules in the formulation even in the unsuitable zeta potential environment[254]. Also, the larger vesicular size of the niosomes incorporated in the hair lotion formulation than the niosomal dispersion may be due to the intercalation of the vesicles in the matrices of HPMC chain[254].

3.7.2 Morphology of cationic niosomes loaded with MM in the hair lotion

Figure 46 showed the TEM images of the cationic niosomes loaded with MM and those incorporated in the hair lotion formulation using the negative staining technique. Niosomes loaded with MM both in niosomal dispersion and hair lotion formulation exhibited spherical shape with the unilamellar vesicular structure. The TEM images of some vesicles that showed the elongated shape may be from the conformation change of the niosomal membrane affected by the neighboring niosomal vesicles[219]. The larger size of niosomes when incorporated in hair lotion formulation

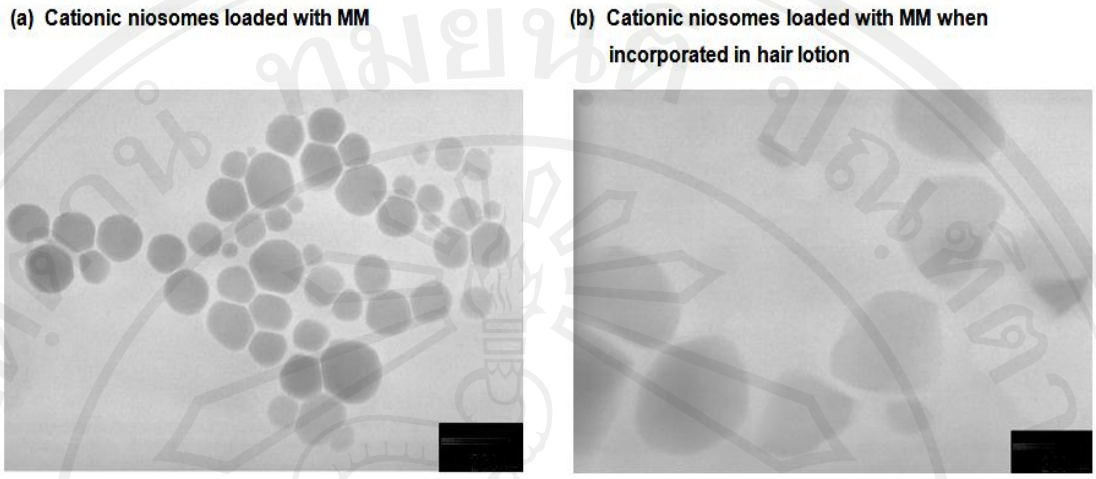


Figure 46 The negative staining TEM images of cationic niosomes loaded with MM (a) and those incorporated in hair lotion (b)

than in the niosomal dispersion may be from the intercalation of the vesicles in the matrices of HPMC which was used as a rheology modifier in the formulation.

3.7.3 Physical stability of cationic niosomes loaded and not loaded with MM when incorporated in the hair lotion

The hair lotion containing MM loaded and not loaded in cationic niosomes stored at all temperatures (4±2, 30±2 and 45±2°C) showed good physical stability without phase separation and sedimentation for 3 months. However, the color of the hair lotions containing both MM loaded and not loaded in niosomes kept at room temperature (30±2) and 45±2°C changed from colorless viscous liquid to pale yellow viscous liquid after 2-month storage, whereas the color change of those kept at 4±2°C was not observed. Since the degradation of α-tocopherol have been caused by various factors, such as oxygen, alkali, light and temperature, thus, the observed color change of hair lotions at high temperature may be from temperature-induced degradation of vitamin E, an ingredient in oil phase of hair lotion formulation[255-256].

The characteristics of the hair lotion containing MM loaded and not loaded in cationic niosomes kept at 4±2, room temperature (30±2) and 45±2°C were presented in Table 19. At initial, the vesicular sizes and zeta potential of the hair lotion containing cationic niosomes loaded and not loaded with MM were 296.50±2.11, 6.47±0.13 and 281.20±1.12 nm, -15.50±0.65 mV, respectively. The pH values of both hair lotions were 4.5, while the polydispersity index indicated the multi-dispersed systems with 0.48±0.07 and 0.39±0.05 for hair lotion containing MM loaded and not loaded in cationic niosomes, respectively. The decreased vesicular sizes of the hair lotion containing cationic niosomes loaded with MM were observed after 3-month storage at all temperatures. However, the vesicular sizes of the formulations containing niosomes loaded with MM were the same as those of the niosomal dispersion at initial (185.20±1.82 nm). For zeta potential, the increased positive charge of the hair lotion composing of MM loaded in niosomes was found at all storage temperatures. The increased values may be from the positive charge effect of the cationic niosomes loaded with MM. Nevertheless, the increased zeta potential values of the formulation containing MM loaded in niosomes were in the unstable range (within ±30 mV)[79]. Since there was no observation of phase separation and aggregation of the hair lotions, the low zeta potential values appeared not to affect the stability of formulations. Also, the addition of HPMC, a rheology modifier, may entangle the molecules of the formulation compositions, resulting in the stable hair lotion.

The less vesicular sizes and zeta potential values of the hair lotion containing MM not loaded in cationic niosomes than at initial were observed after 3 months at all storage temperatures. The decreased vesicular sizes of both hair

Table 19 Characteristics of MM loaded and not loaded in cationic niosomes when incorporated in hair lotions when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

Formulation	pH		Z-zeta potential (mV)		Particle diameter (nm)		Polydispersity index	
	Initial	3 months	Initial	3 months	Initial	3 months	Initial	3 months
Hair lotion containing MM loaded in cationic niosomes								
45±2°C	4.5	4.5	6.47±0.13	11.57±0.12	296.50±2.11	205.47±3.12	0.48±0.07	0.54±0.01
RT (30±2°C)	4.5	4.5	6.47±0.13	11.93±0.78	296.50±2.11	207.80±0.23	0.48±0. 07	0.56±0.03
4±2°C	4.5	4.5	6.47±0.13	12.77±0.41	296.50±2.11	187.07±1.68	0.48±0. 07	0.46±0.04
Hair lotion containing MM not loaded in cationic niosomes								
45±2°C	4.5	4.5	-15.50±0.65	-32.80±1.14	281.20±1.12	204.39±1.59	0.39±0.05	0.26±0.00
RT (30±2°C)	4.5	4.5	-15.50±0.65	-27.47±1.73	281.20±1.12	169.10±3.43	0.39±0.05	0.22±0.01
4±2°C	4.5	4.5	-15.50±0.65	-28.10±0.79	281.20±1.12	162.13±3.22	0.39±0.05	0.26±0.01

lotions containing MM loaded and not loaded in cationic niosomes may be from the HPMC polysaccharide gel. As known, the gel structure of HPMC is formed when heated at the gelation temperature between 50-90°C[257]. After reaching the viscosity temperature, the viscosity of HPMC solution will decrease when the temperature increases to the thermal gel point[258]. When the intercalation of HPMC and the compositions of hair lotion decreased, the vesicular sizes of the hair lotions appeared to be less than at initial. For the zeta potential of the hair lotion containing MM not loaded in cationic niosomes, the decreased zeta potential values of formulations were observed, owing to the degradable compound of MM. As MM was incorporated in hair lotion composed of water of about 80%, the hydrolysis reaction of MM, a methyl ester of myristic acid, to myristic acid might occur in the acidic environment (pH of formulation was 4.5)[56].

3.7.4 Chemical stability of MM loaded and not loaded in cationic niosomes incorporated in the hair lotion

Figure 47 demonstrated the percentage remaining of MM in the hair lotions containing MM loaded and not loaded in cationic niosomes when kept at 4±2, room temperature (30±2) and 45±2°C for 3 months. After 3 months, the percentages remaining of MM in the hair lotion containing MM loaded in niosomes were higher than that not loaded in niosomes at all storage temperatures. This may be due to the protection of MM when loaded in niosomes in which the loaded hydrophobic molecules (MM) are intercalated with other compositions of the vesicular membrane, such as non-ionic surfactant, cholesterol and the charged lipid (DDAB), and are shielded from chemical reaction[71,220,236]. However, the decreased remaining amounts of MM both in loaded and not loaded in cationic niosomes were observed when the

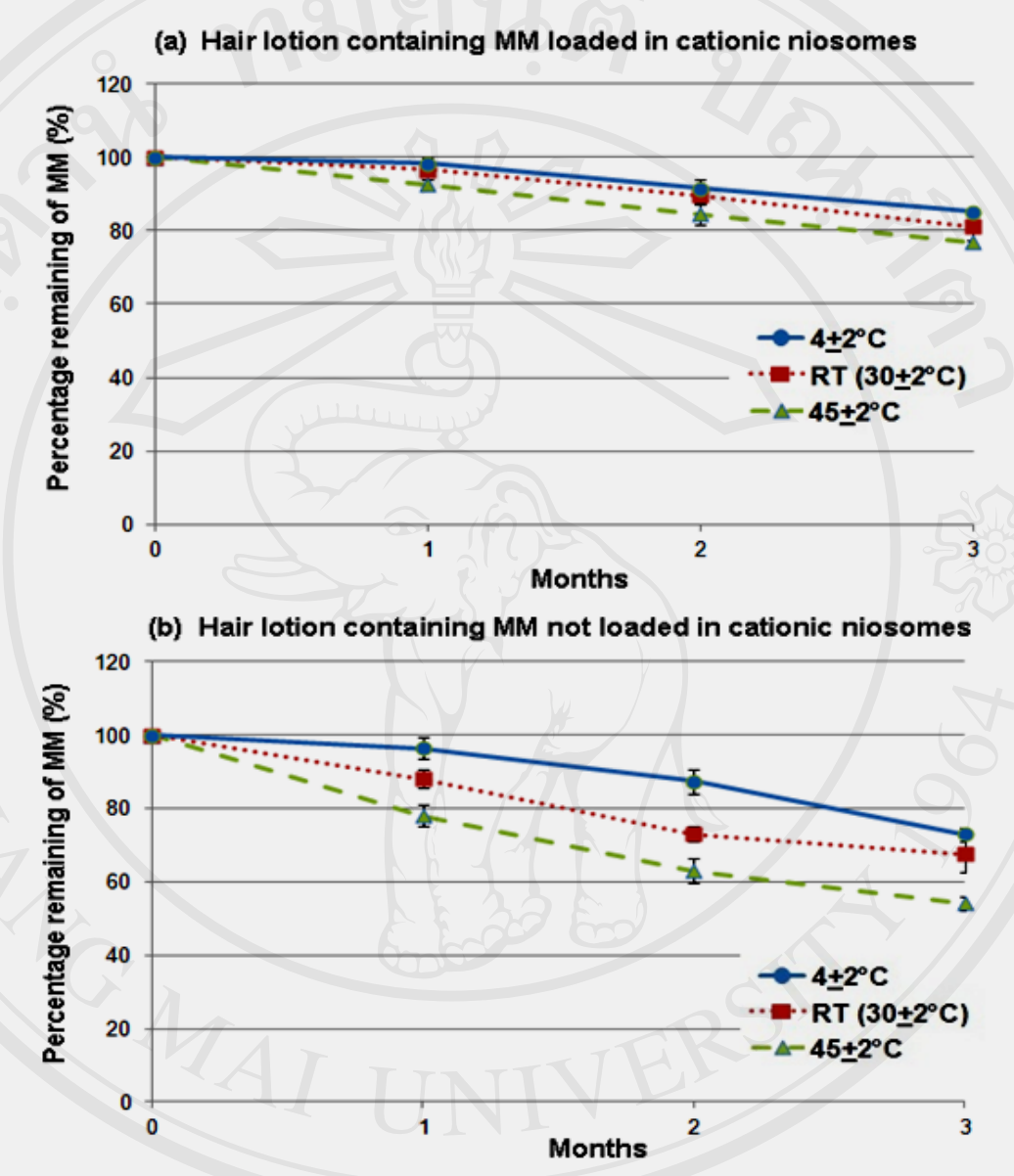


Figure 47 Percentage remaining of MM loaded (a) and not loaded (b) in cationic niosomes in the hair lotions when kept at 4±2, room temperature (RT; 30±2) and 45±2°C for 3 months

storage period and temperature increased. The low storage temperature at 4±2°C gave higher stability of MM both in loaded and not loaded in cationic niosomes in the hair lotions than the other two storage temperatures (room temperature;30±2 and 45±2°C). The chemical stability of the hair lotion consisted of MM loaded in cationic niosomes was higher than that consisted of the free form MM. Water containing in the hair lotion may hydrolyze MM in an acidic environment, resulting in the occurrence of the degradable compound, myristic acid[56].

3.8 *In vitro* transfollicular penetration and *in vivo* rabbit skin irritation and melanogenesis induction of the hair lotion containing MM loaded and not loaded in cationic niosomes

3.8.1 *In vitro* transfollicular penetration of the hair lotion containing MM loaded and not loaded in cationic niosomes

Currently, the pilosebaceous unit consisted of hair follicle, sebaceous gland, arrector pili muscle and hair shaft has been increased attraction on percutaneous absorption due to high vascularization region as well as no interrupted barrier for permeation in the follicle[10,113]. The evaluation of *in vitro* transfollicular penetration has been developed and intensely investigated[10,112]. The selective follicular blocking by nail varnish and differential stripping combined tape stripping and cyanoacrylate skin surface stripping have been demonstrated as the non-invasive methods for investigation of follicular penetration[156,158]. The porcine skin has been reported to have the morphology, function and penetration of topically applied substances similar to the human skin[233-234]. In this study, the excess sebaceous fat of porcine skin was removed by the pinching method that did not affect the hair follicles during the

procedure[235-236]. Two skin systems, including opened and blocked hair follicle skin, were prepared for the evaluation of follicular penetration of MM loaded and not loaded in cationic niosomes when incorporated in the hair lotion formulations. The topical penetration of MM through the opened hair follicle skin mounted on vertical Franz diffusion cells was mediated via both transepidermal and follicular routes, while the penetration through the blocked hair follicle skin was mediated only through the transepidermal route. The different flux between the opened hair follicle skin and blocked hair follicle skin was determined as the transfollicular penetration flux[236].

The cumulative amounts of MM in skin and receiver compartment at 1, 2, 4 and 6 h were presented in Fig. 48. The cumulative amounts, fluxes and follicular penetration per one hair follicle by the follicular closing technique using vertical Franz diffusion cells at 6 h of MM loaded and not loaded in cationic niosomes when incorporated in hair lotion formulations were in Table 20. The cumulative amounts of MM from two hair lotion formulations (the compositions of base formulation No. 12 were as following: non-ionic surfactant HC-60, jojoba oil, vitamin E, trimethyl glycine, panthenol, glycerol, propylene glycol, liquid germall plus, HPMC and water) through skin were increased with the increased time. In the skin after 6 h, the cumulative amounts of MM loaded in cationic niosomes incorporated in hair lotion formulations (20.4586 $\mu g/cm^2$) exhibited higher amounts than that not loaded in cationic niosomes (14.1260 $\mu g/cm^2$) of about 1.45 times. The cumulative amounts of MM in the receiver compartment from both hair lotion formulations were similar to that observation in the skin. At 6 h, MM loaded in cationic niosomes in hair lotion formulations (1.9910 $\mu g/cm^2$) gave higher cumulative amounts than that not loaded in cationic niosomes (1.5130 $\mu g/cm^2$) of 1.32 times in receiver chamber. As known,

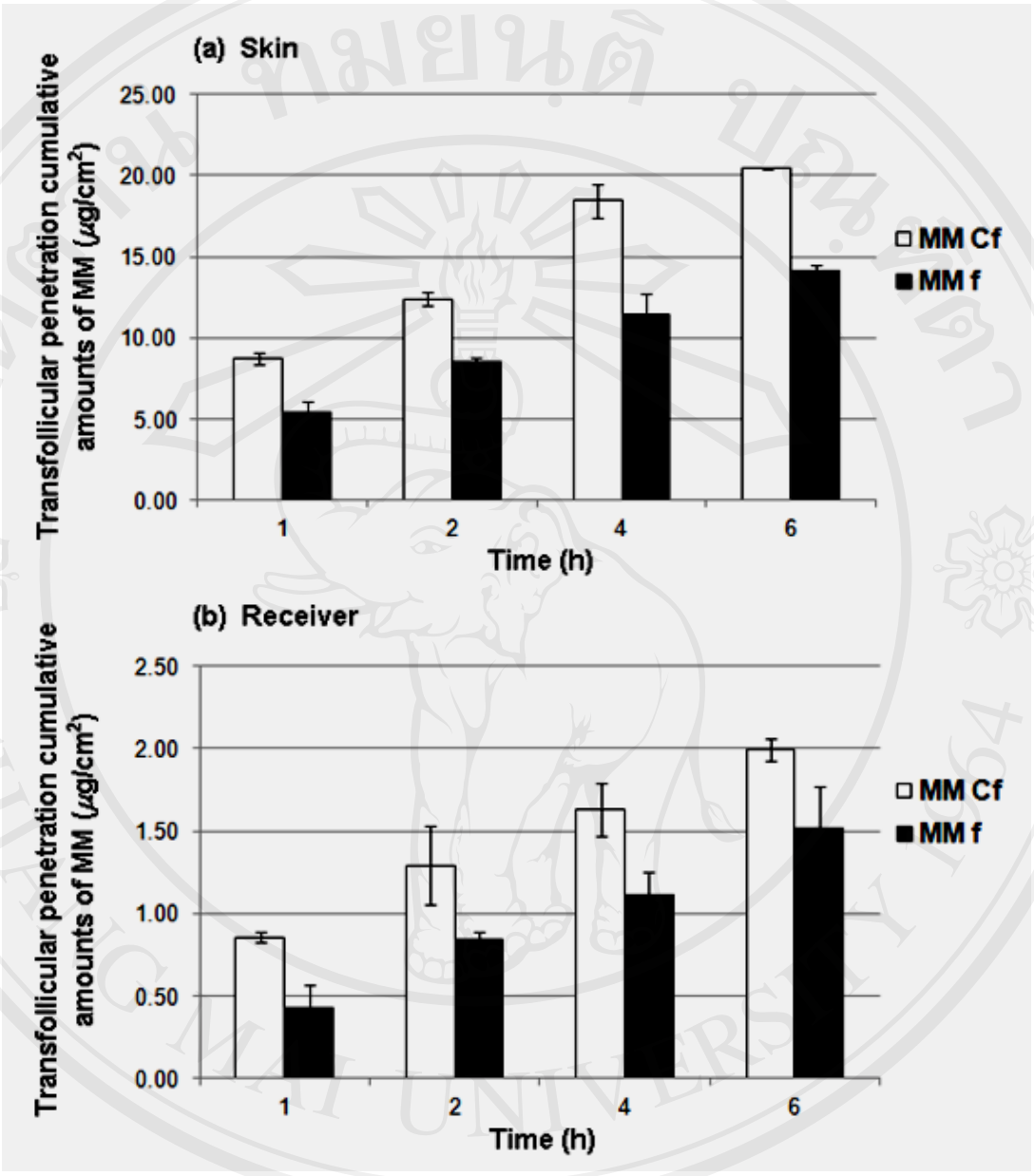


Figure 48 Cumulative amounts in skin (a) and the receiver (b) of MM (μg/cm^2) loaded (MM Cf) and not loaded (MM f) in cationic niosomes incorporated in hair lotion by follicular closing technique using vertical Franz diffusion cells at 0, 1, 2, 4 and 6 h

Table 20 The cumulative amounts (μg/cm^2), fluxes (μg/cm^2/h) and follicular penetration per one hair follicle (μg/one hair follicle) of MM loaded and not loaded in cationic niosomes incorporated in hair lotions by follicular closing technique using vertical Franz diffusion cells at 6 h

Formulation	Skin			Receiver		
	Cumulative amounts (μg/cm^2)	Fluxes (μg/cm^2/h)	Follicular penetration per one hair follicle (μg/one hair follicle)	Cumulative amounts (μg/cm^2)	Fluxes (μg/cm^2/h)	Follicular penetration per one hair follicle (μg/one hair follicle)
Hair lotion containing MM loaded in cationic niosomes (MM Cf)	20.4586	3.4098	0.2447	1.9910	0.3318	0.0238
Hair lotion containing MM not loaded in cationic niosomes (MM f)	14.1260	2.3543	0.1563	1.5130	0.2522	0.0167

liposomes and niosomes, the closed vesicles loaded with hydrophilic as well as lipophilic compounds, have been indicated their effective compound delivery for topical and systemic administrations[9,75,95,238]. MM loaded in cationic niosomes when incorporated in hair lotion exhibited more effective transfollicular penetration than that not loaded in cationic niosomes. Also, propylene glycol and surfactant have been proved to enhance transfollicular penetration[9,53]. These two substances were selected to incorporate in the hair lotion formulation for follicular penetration enhancement. The results found that follicular penetration of MM loaded and not loaded in cationic niosomes when incorporated in the hair lotions was higher than that loaded in cationic niosomal dispersion and in solution of about 2 times both in skin and receiver compartment.

The particle size as well as the surface charge appeared to have the influence on the relative penetration depth of transfollicular penetrants[136]. Follicular melanocytes that are responsible for the hair pigment production locate in the hair follicles at matrix cell and bulge region. The particle size reached the bulge region should be in the range of 230-643 nm[136]. For the surface charge, the amphoteric and cationic charge of liposomes entrapped with carboxyfluoresceine dye demonstrated the penetration depth of dye in the full hair follicle of approximately 70%[240]. Thus, the 300-nm positively charge niosomes loaded with MM when incorporated in hair lotion may penetrate into the hair follicle and subsequently stimulate the production of melanin for the treatment of canities.

3.8.2 *In vivo* rabbit skin irritation by the single closed patch test

The irritant contact dermatitis (skin irritation) has been demonstrated to be the most frequent inflammatory response of skin occurred after contact to irritant.

Irritant is any compound that can cause cell damage after contacting for sufficient time and concentration. In fact, physicochemical properties of compounds cannot exactly be used to predict the irritation induced potential[175]. The *in vivo* rabbit skin irritation, an adopted standard method used for skin irritation analysis[176], was used to evaluate the skin irritation of the MM systems.

The skin irritation of MM in various systems, including MM solution in ethanol (1 mg/ml) and MM loaded in cationic niosomes, MM loaded and not loaded in cationic niosomes incorporated in hair lotions, and the control systems, which were ethanol (solvent), blank cationic niosomes and hair lotion base, was performed by the single closed patch in comparing to the positive irritant (20% w/w sodium lauryl sulfate), distilled water and the untreated area. The positive irritant showed slight irritation with the PII value of 1.84, while MM solution and solvent (ethanol) indicated slight irritation with the low PII values of 0.33 and 0.25, respectively (Table 21). Both blank cationic niosomes and MM loaded in cationic niosomes slightly caused irritation after the 1 h-patch removal. However, the slight irritation of both blank cationic niosomes and MM loaded in cationic niosomes with the same PII value of 0.17 was not observed after the 96 h-patch removal (4^{th} day). For hair lotion formulations, the slight irritation with the PII values of 0.50, 0.08 and 0.08 were observed from MM loaded and not loaded in cationic niosomes incorporated in the hair lotion and the hair lotion base, respectively. However, the hair lotion formulation induced irritation disappeared at the 4^{th} day after patch removal, while skin irritation of sodium lauryl sulfate was not found at 5^{th} day after patch removal.

The previous *in vitro* cytotoxicity assay in normal human skin fibroblasts of MM, blank cationic niosomes and MM loaded in cationic niosomes demonstrated

Table 21 The *in vivo* rabbit skin irritation of MM solution, solvent (ethanol), blank cationic niosomes and MM loaded in cationic niosomes, hair lotion base and hair lotions containing MM loaded and not loaded in cationic niosomes, the untreated area, distilled water and sodium lauryl sulfate by the single closed patch test

Sample	PII value	Category of irritation degree based on PII value
Solvent (ethanol)	0.25	slight irritation
MM (1 mg/ml in ethanol)	0.33	slight irritation
Blank cationic niosomes	0.41	slight irritation
MM loaded in cationic niosomes	0.50	slight irritation
Hair lotion base	0.08	slight irritation
Hair lotion containing MM not loaded in cationic niosomes (0.7 mg/ml)	0.08	slight irritation
Hair lotion containing MM loaded in cationic niosomes (0.7 mg/ml)	0.50	slight irritation
Untreated area	0.00	non-irritation
Distilled water	0.00	non-irritation
Sodium lauryl sulfate (20% w/w)	1.84	slight irritation

slight and moderate cytotoxicity of 97.25$\pm$2.30, 73.81$\pm$2.86 and 73.20$\pm$3.49% cell viability, respectively. For *in vivo* rabbit skin irritation assay, the observed slight irritation was in agreement with the cytotoxicity assay in cell culture. However, the higher irritation response of MM *in vivo* than *in vitro* may be the effect from the solvent (ethanol) used for solubilization. *In vitro* skin irritation of ethanol in human keratinocytes using the release of proinflammatory mediators and cell viability has been indicated the mild irritation[259]. The higher PII value of the hair lotion containing MM loaded in cationic niosomes than the hair lotion containing MM not loaded in cationic niosomes and the base formulation may be from the direct contact of the MM

when loaded in niosomes with the skin.

3.8.3 *In vivo* melanogenesis induction in aged mice

A. Melanogenesis induction evaluation

Figure 49 showed the pigmentation scores in mice (n = 3 per group) treated with the hair lotions containing theophylline, MM loaded and not loaded in cationic niosomes and the base formulation. As known, theophylline that stimulates melanin formation through cAMP pathway as well as the increased gamma-glutamyl transpeptidase- and tyrosinase- reactive cells has been used as a standard reference *in vitro* melanogenesis induction study[41, 206-207]. Thus, theophylline was selected to incorporate in the hair lotion (0.5 mg/ml) and used as a positive control for the *in vivo* melanogenesis induction evaluation. The bluish spot that exhibited the first sign of skin and hair pigmentation was observed in the system of hair lotions containing theophylline and MM loaded and not loaded in cationic niosomes after 14 days of application, whereas the change of skin color and hair growth in hair lotion base was found after 21 days of application. The peak of skin and hair pigmentation was first observed in hair lotion containing MM loaded in cationic niosomes, followed by hair lotions containing MM not loaded in cationic niosomes and theophylline, respectively. The first peak observation of skin and hair pigmentation in the system of hair lotion containing MM loaded in cationic niosomes may be from the enhanced penetration of niosomes loaded with MM. The peak of pigmentation in hair lotion containing theophylline was found after 56 days of application, while only white hairs without skin color changes were found in hair lotion base. The delayed effect of theophylline for pigmentation induction may be from the age of the mice that the level of skin tyrosinase activity is generally observed in the young 30- to 35-day-old mice

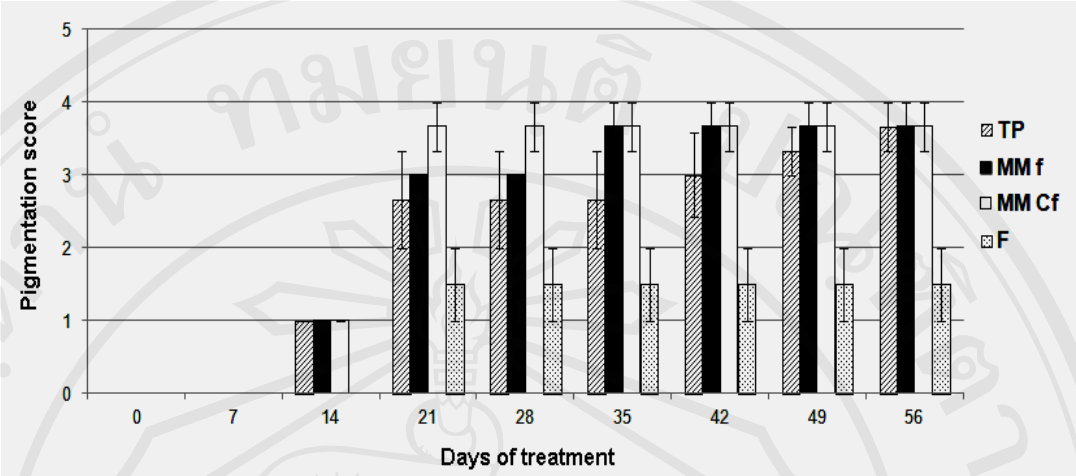


Figure 49 Pigmentation scores in mice (*n*=3 per group) treated with hair lotions containing theophylline (TP), MM loaded (MM Cf) and not loaded (MM f) in cationic niosomes, and hair lotion base (F)

more than the aged 6-month-old mice. Theophylline has been exhibited effect on the increased skin coat darkness and skin tyrosinase activity in the young mice, but not in the aged mice[260]. The aged mice used in this study mimick the aged people that often have canities from the aged process and subsequently leads to the exhaustion of melanin production[22].

B. Histological examination

The skin specimen after treatment with hair lotions containing theophylline, MM loaded and not loaded in cationic niosomes, and the base formulation were presented in Fig. 50. Melanin bleach staining technique is used to investigate for melanin. Generally, cell observation is obscured in the presence of high amounts of melanin pigment. The ability to be bleached of melanin has been used to identify melanin[261]. The alterations in skin, melanization and hair follicle formation were significantly observed in mice treated with the hair lotions containing

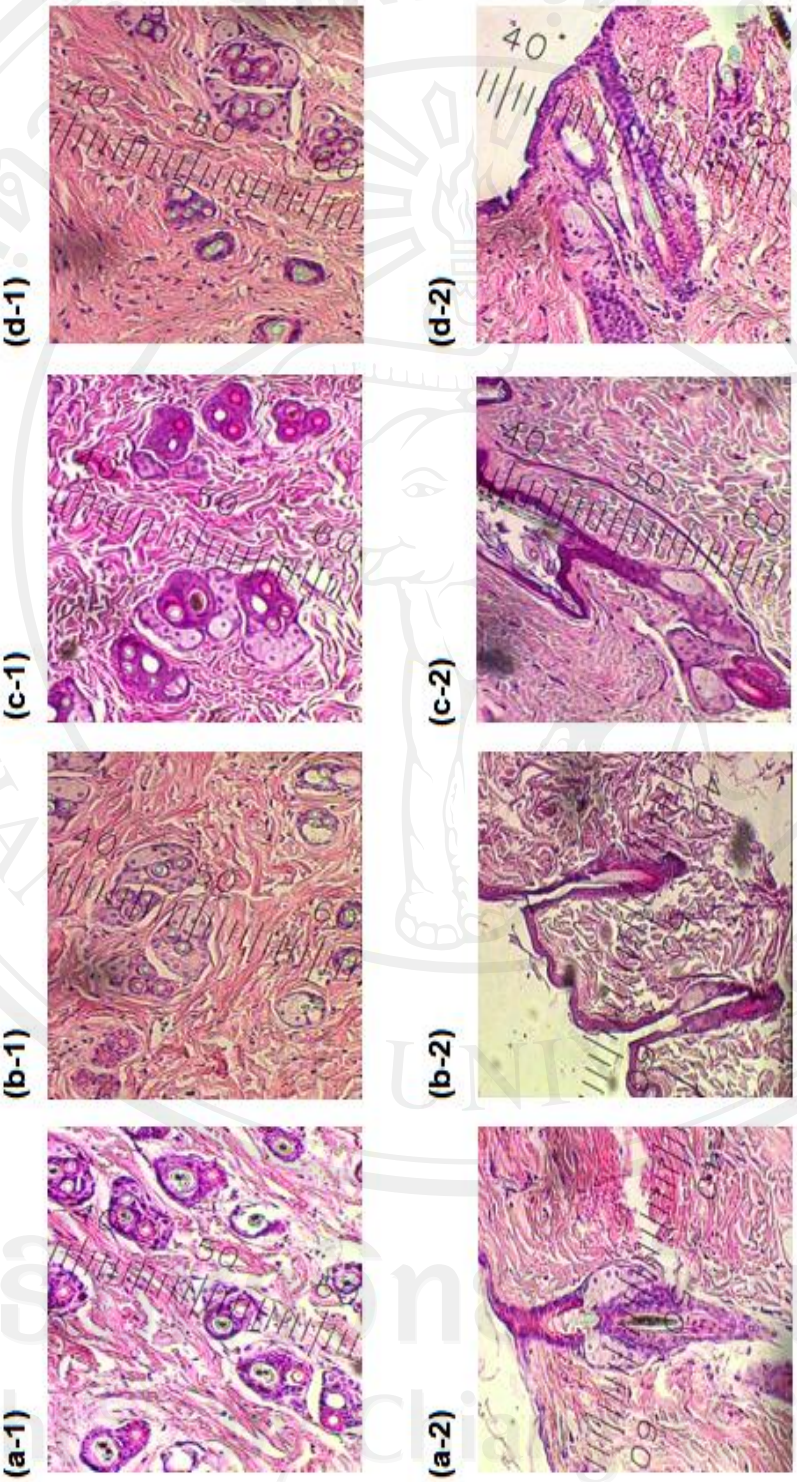


Figure 50 Histological examination of skin specimens treated with hair lotions containing theophylline (a), MM loaded (b) and not loaded (c) in cationic niosomes, and hair lotion base (d) using melanin bleach staining technique. 1 and 2 represented the cross-sectional and longitudinal views, respectively.

theophylline and MM loaded and not loaded in cationic niosomes, while the hair follicle formation with few melanization was found in mice treated with the base formulation (Fig. 50, a1-d1). The wall of the new hair follicle containing the migratory melanocytes was also well detected in the system of the hair lotions containing theophylline and MM loaded and not loaded in cationic niosomes. In the longitudinal view, the new hair follicle development with high contents of melanin was found in mice treated with the hair lotion containing theophylline and MM loaded and not loaded in cationic niosomes, while the follicle with little melanin formation was observed in the base formulation (Fig. 50, a2-d2). The difference of the histological examination of theophylline and MM loaded and not loaded in cationic niosomes was not significantly observed. This may be resulted from the peak pigmentation response of all samples at the time of histological examination.