CHAPTER IV

RESULTS AND DISCUSSION

4.1 Determination of antioxidant activity of marigold flower extracts

4.1.1 Scavenging effect on 1, 1-diphenyl-2-2picrylhydrazyl (DPPH) radical

As we know that the polyphenolic compounds contained in plant extracts possessed the antioxidant activity of the extracts due to their ability to be donors of hydrogen atom or electrons to capture the free radicals.[25, 49] In this method, DPPH assay, the antioxidant activity of the extracts was measured by UV- Visible spectrophotometer at 520 nm after the DPPH radicals had scavenged by the compounds that have antioxidant ability for 30 min corresponds inversely to the remaining DPPH radicals present.

The DPPH radical scavenging activities of marigold flower extracts (**H**, **EA**, **Et** extract) and fractions (F7-F14) compared with reference antioxidants (trolox, quercetin and gallic acid) are shown in Figure 4.1. From this assay, the F1-F6 fractions showed very low activity (data not shown).

In the part of marigold flower extracts obtained from continuous extraction, ethyl acetate (**EA**) extract showed the highest antioxidant activity with IC_{50} of 15.71 ± 1.45 µg/ml followed by ethanol (**Et**) extract (IC_{50} of 71.09 ± 7.24 µg/ml) and hexane (**H**) extract (353.18 ± 37.92 µg/ml). Each solvent can extract different compounds from marigold flower resulting in different antioxidant activity. The polarity indexes

of hexane, ethyl acetate and ethanol are 0, 4.4 and 5.2, respectively. According to ethyl acetate extract exhibits the highest antioxidant activity and the polarity index is higher than hexane but lower than ethanol so these can suggest that both of polar and semi-polar compounds such as phenolic compounds played the major role in the radical scavenging activity of marigold flower. However, these presented lower antioxidant activity than pure reference standards; trolox (IC_{50} 9.32 ± 1.19 µg/ml), quercetin (IC_{50} 5.07 ± 0.40 µg/ml) and gallic acid (IC_{50} 2.61±0.30 µg/ml). For semi-purified fractions obtained from **EA** extract, interestingly found that the fraction 9 (F9) and fraction8 (F8) exhibited higher antioxidant activity than trolox but lower than quercetin and gallic acid with IC_{50} 7.64 ± 0.10 and 7.80 ± 0.49 µg/ml, respectively. F9 and F8 fractions were obtained by using the mixture of ethyl acetate and hexane (70:30) in fractionation so that they should have both polar and semi-polar compounds. This result is concomitant with the result of crude extracts so this is strongly suggests that both of polar and semi-polar compounds played the major role in the radical scavenging activity of marigold flower.

4.1.2 Thiobarbituric acid reactive substances species (TBARS) assay

Lipid peroxidation, a typical free radical oxidation, it has been suggested to be an important cause in cellular damage which is strongly associated with skin aging, carcinogenesis and other diseases. The artificial biomembranes, liposome, have been used as a model system for in vitro lipid peroxidation studies.

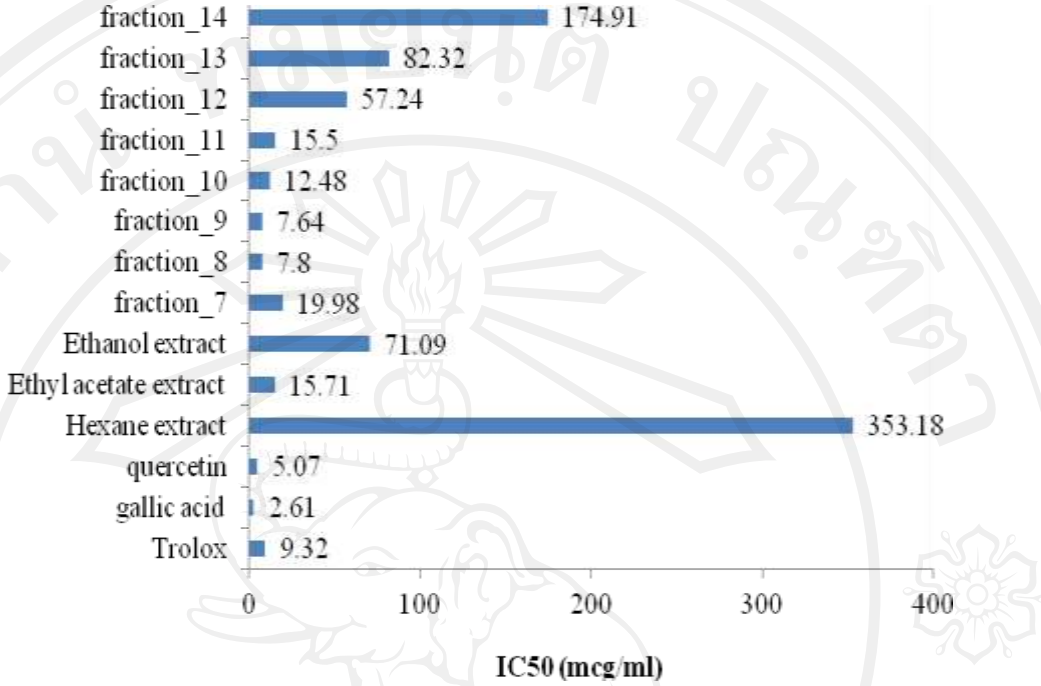


Figure 4.1 The IC_{50} values (µg/ml) of marigold flower extracts and fractions from **EA** extract by DPPH assay

This method, known as Thiobarbituric acid reactive substances species (TBARS) assay, concerns the spectrophotometric measurement of pink pigment which produced through the reaction of thiobarbituric (TBA) with malondialdehyde (MDA), secondary lipid peroxidation product. The evaluation of the absorbance at 540 nm gives a measure of the extract of the lipid degradation.

The antioxidant activities of marigold flower extracts (**H**, **EA** and **Et** extracts) and fractions (F7-F14) compared with reference antioxidants (trolox, quercetin and gallic acid) are shown in Figure 4.2. From this assay, the F1-F6 fractions showed very low activity (data not shown).

The **EA** extract showed the highest antioxidant activity with IC_{50} of 91.48 ± 0.35 µg/ml followed by **Et** extract (IC_{50} of 263.80 ± 35.88 µg/ml). For **H** extract, we

cannot calculate IC_{50} from the graph plotted inhibition against extract concentration because %inhibition of the highest concentration of hexane extract in ethanol does not reach 50%. Comparison with reference antioxidants, the **EA** extract showed lower antioxidant activity than quercetin (IC_{50} of 18.65 ± 1.13 µg/ml) and gallic acid (IC_{50} of 45.95 ± 18.59 µg/ml) acid but about 2 times higher than trolox (IC_{50} of 210.47 ± 3.67 µg/ml) while the ethanol extract showed lower activity than gallic acid and quercetin but comparable to trolox. The antioxidant activity from this method expressed the same result obtained from DPPH assay; **EA** >**Et** >**H** extracts. For semipurified fractions obtained from **EA** extract, the F9 and F8 fractions also exhibited higher antioxidant activity than trolox but lower than quercetin and gallic acid with IC_{50} 38.95 ± 0.42 and 42.73 ± 0.42 µg/ml, respectively, which is similar to that found in DPPH assay and can be explained with same reason**.** However, F9 and 8 fractions were about 5 times higher activity than trolox and comparable to gallic acid.

The antioxidant activity of marigold flower extracts from these two assays, DPPH and TBAR assay, showed us the same pattern despite they have the different mechanism of action. But as previously described, the used of different methods and concentrations are necessary in antioxidant activity assessment. A sample possessed DPPH free radical scavenging property indicated that its mechanism of action was hydrogen donor and terminated the oxidation process by converting free radicals to more stable product. While TBAR assay presented that its mechanism was the ability to inhibit the lipid peroxidation reaction of the samples. The combination of two difference methods, applied in this study, was a good choice to determine the antioxidant activity of marigold extracts.

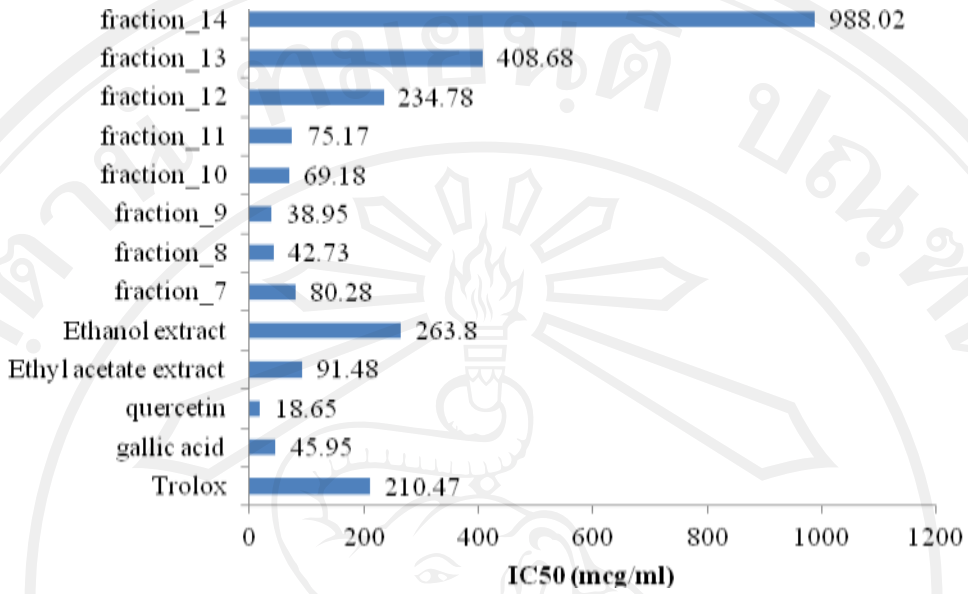


Figure 4.2 The IC_{50} values (µg/ml) of marigold flower extracts and fractions from **EA** extract by TBARS assay.

4.2 Determination of Total Phenolic content in marigold flower extracts

The total phenolic contents of marigold crude extracts and fractions are shown in Figure 4.3. From the result, **EA** extract showed the highest total phenolic content (318.05 ± 1.00 mg gallic acid/g extract) compared with other marigold flower extracts (**H** and **Et** extracts). While F8 and F9 fraction expressed the highest total phenolic contents (518.50 ± 2.05 and 461.7 ± 5.91 mg gallic acid/g extract) compared with those fractions.

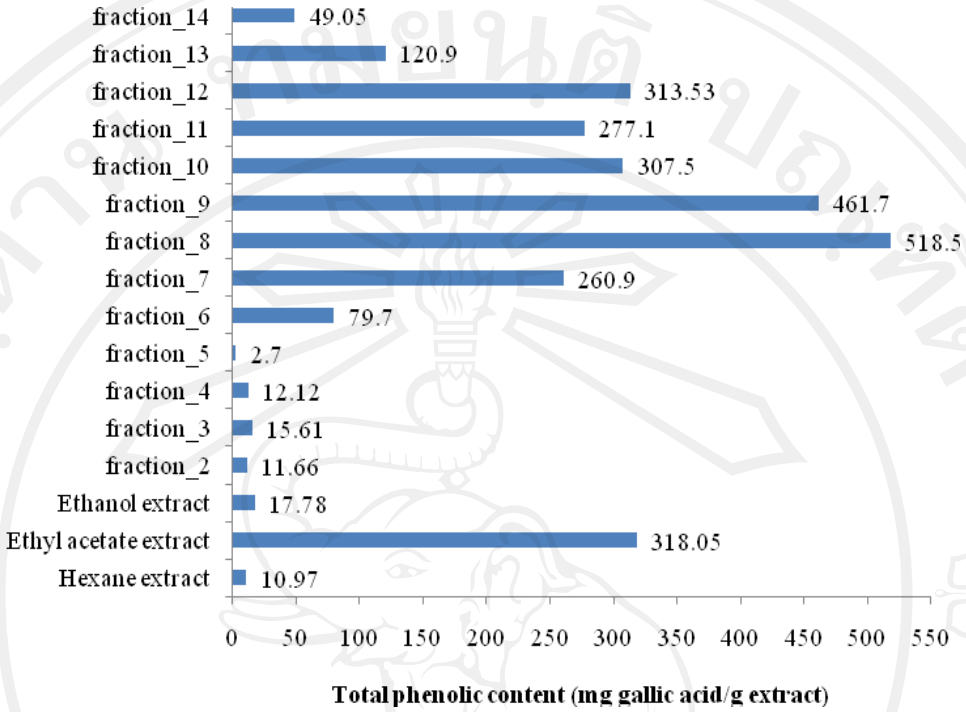


Figure 4.3 The total phenolic contents of marigold flower extracts and fractions from EA extracts

In general, most phenolic compounds reveal some antioxidant activity. Therefore, extracts with higher phenolic contents would show higher antioxidant activity. From our results in Table 4.1, EA, F8 and F9 fractions which revealed the highest antioxidant activity also showed the highest total phenolic contents among extracts and fractions. This result indicates that phenolic compounds are major contributors to the antioxidant activity of the marigold flower extracts.

Table 4.1 Total phenolic and antioxidant activities of marigold flower extracts and fractions from EA

Sample	Total phenolic (mg GAE/g extract)	IC_{50}(µg/ml)	
		DPPH assay	TBARS assay
Hexane extract	10.97±0.12	353.18±37.92	LA
Ethyl acetate extract	318.05±1.00	15.71 ± 1.45	91.48±0.35
Ethanol extract	17.78±1.68	71.09±7.24	263.80±35.88
Fraction 2	11.66±0.04	LA	LA
Fraction 3	15.61±0.02	LA	LA
Fraction 4	12.12±0.01	LA	LA
Fraction 5	2.70±2.03	LA	LA
Fraction 6	79.70±2.03	LA	LA
Fraction 7	260.90±2.05	19.98 ±1.13	80.28±1.89
Fraction 8	518.50±2.05	7.80±0.49	42.73±0.14
Fraction 9	461.70±5.91	7.64±0.10	38.95±0.42
Fraction 10	307.50±0.00	12.48±2.91	69.18±7.13
Fraction 11	277.10±3.74	15.50 ±4.14	75.17±1.77
Fraction 12	313.53±3.91	57.24±0.68	234.78±16.74
Fraction 13	120.90±2.00	82.32±0.69	408.68 ±38.17
Fraction 14	49.05±5.00	174.91±25.58	988.02±46.60
Trolox	-	9.32±1.19	210.47±3.67
Gallic acid	-	2.61±0.30	45.95±18.59
Quercetin	-	5.07±0.40	18.65±1.13

LA= Low activity

4.3 Formulation, characterization and stability of unloaded nanostructured lipid carriers (unloaded NLC)

The unloaded NLC formulations prepared in this study can classify into 3 groups: formulation A (A1-A9), formulation B (B1-B6) and formulation C (C1-C6). The compositions of each formulation were previously mentioned in Chapter III. All formulations were prepared by the same equipments and method. After the preparation, the mean particle size, size distribution, zeta potential and physical property of unloaded NLC were observed.

First, consideration for formulation A (A1–A9), the results revealed that the presence of SA in the formulation (A2, A5 and A8) affected the mean particle size of unloaded NLC when compared with formulations without SA at the same ratio of solid lipid: liquid lipid (A1, A4 and A7, respectively). This is due to the alkyl chain length of SA molecules and its acidic property. The mean particle size of A5 was larger than A4 (172.5 and 123.4nm). A8 (125.6nm) was slightly smaller than A7 (150.0nm) while A1 and A2 was not different (124.4 and 124.3nm). The polydispersity index (PDI) was in the range of 0.139 – 0.403 indicate the narrow size distribution of particles. The pH of formulation with SA was slightly decreased. (data not shown).

The comparison between formulation A with CCM (A3, A6 and A9) and those without CCM (A1, A4 and A7, respectively), the results revealed that the presence of CCM affected the physical property of NLC because some particles were optically observed in A3, A6 and A9 after 24 hours of preparation. The mean particle size when compared between the formulations with the same ratio of solid lipid: liquid

lipid was not the same pattern. The mean particle size of A3 and A6 (224.8 and 159.1nm) were larger than A1 and A4 (124.4 and 123.4nm) while A9 (138.4nm) was smaller than A7 (150.0nm). The PDI were in the range of 0.164-0.403. For the effect of solid lipid: liquid lipid ratio, formulation A1 (ratio 2:1), A4 (ratio 1:1) and A6 (ratio 1:2) were investigated. The results showed that the increasing of liquid lipid content in formulation (A4 and A7) leaded NLC to translucent and jelly- like dispersion. The mean particle size of A4 (123.4nm) and A1 (124.4 nm) were smaller than A7 (150.0nm). At this time, the optimal formulation for unloaded NLC from formulation A is A1 (ratio of solid lipid: liquid lipid was 2:1, without SA and CCM). The PDI ranged from 0.139-0.403 by A4 showed the highest value (0.403). The absolute zeta potential values of A1-A9 were in the range of -33.5 to -50.3 mV, showed that NLC dispersion should possess a good physical stability.

Second, formulation B (B1-B6), we decided to exclude the formulations with CCM as liquid lipid because in the previous study of formulation A, the small particles were optically observed in all formulation with CCM. To compare the formulations B with and without SA, the result revealed that some particles were optically observed from formulations without SA: B1 and B3 except B5 whereas B2, B4, and B6 with SA were not. The mean particle sizes of formulations with SA were smaller than formulations without SA at all ratios of solid lipid: liquid lipid. The pH of formulations with SA was slightly decreased due to fatty acid property of SA same as formulation A. Formulation B with different solid lipid: liquid lipid ratio did not show the difference in appearance of NLC dispersion but showed the difference in the mean particle size values. The mean particle size of B4 was larger than B2 and B6 (198.8, 150.3 and 150.2nm, respectively). The absolute zeta potential values of B1-

B6 were in the range of 36- 45 mV. For the formulation A at solid lipid: liquid lipid ratio were 1:1 and 1:2, we observed the translucent jelly-like dispersion but formulation B they were normally white dispersion due to the property of liquid lipid used in each formulation.

Usually, particle aggregation is less likely to occur for charged particles with high zeta potential ($>|30|$ mV) due to electric repulsion. Though this rule cannot be strictly applied in the presence of Tween® and Span®; steric stabilizer, which can decrease the zeta potential. However, the observed zeta potential values of formulation A and B were in range -30.2 to -51.2mV suggested that NLC possessed good stability during storage. [50-53]

Third, the last formulation that has been investigated consists of Poloxamer 188® as surfactant, GB as solid lipid, widely used to prepare NLC in many studies, and GC as liquid lipid. From this formulation (C1-C6), we obtained the white homogenously emulsion but they were more viscous after 24 hours of production. The smallest NLC dispersion form formulation C was obtained from C6 (446.7nm) while the mean particle size values of C1-C5 were almost larger than 1000nm. Even though C6 showed the smallest mean particle size among formulation C but it was significantly larger than the formulation A and B. The PDI of all formulation C were in the range of 0.45-0.83 indicated that the highly distribution of nanoparticles or the aggregation of particles had occurred. In addition to the smallest size, C6 also showed the lowest viscosity of formulation C.

From the characterization study performed at 24 hr (1 day) after the production, the formulations A1 (solid lipid: liquid lipid=2:1), B2 (solid lipid: liquid lipid=2:1) and B6 (solid lipid: liquid lipid =1:2) showed the optimal particles, PDI,

zeta potential and acceptable appearance that were considered for further development of ME-NLC.

Many formulations with inappropriate appearance (aggregation of particles, creaming or gelation) were excluded from the characterization study of NLC dispersion at 1 day after preparation however all of them were stored at room temperature in a well-tight container and protected from light for three months to investigate their stability. The physicochemical property was observed at 30, 60 and 90 days after preparations. The result in Table 4.2 revealed that some excluded formulations showed the nanosize particle. For example A9, we observed the small particle in dispersion but the result of particle size at day30, 60 and 90 were not exceed the nanometer range (158.7, 162.8 and 169.8nm, respectively). After consider to the size distribution curve of A9 at day30, it showed a second small peak of large particles around 2,000-3,000 nm indicated to some particles optically observed. For the stability of formulation A1, B2 and B6, they showed small particle size and size distribution, high zeta potential and good physical stability with absence of particle aggregation or phase separation at all predetermined time intervals (30, 60 and 90 days after preparations). This stability test result confirmed that these 3 formulations were appropriate for further development.

Table 4.2 The mean particle size, polydispersity index (PDI) and zeta potential value of unloaded NLC dispersion after storage for 30, 60 and 90 days.

Formulation		**Particle size (nm)**	**PDI**	**Zeta potential (mV)**
A1	Day1	124.4±2.135	0.223±0.021	-39.5
	Day30	157.3±3.465	0.400±0.033	-40.1
	Day60	161.8±1.586	0.188±0.007	-45.4
	Day90	199.8±2.319	0.216±0.004	-39.2
A2	Day1	124.3±0.825	0.163±0.035	-36.1
	Day30	152.7±1.646	0.144±0.003	-36.0
	Day60	177.1±2.500	0.145±0.013	-37.5
	Day90	217.2±2.727	0.127±0.019	-35.7
A3	Day1	224.8±3.859	0.403±0.042	-38.3
	Day30	155.8±0.957	0.212±0.017	-38.3
	Day60	163.6±5.434	0.319±0.059	-39.8
	Day90	162.5±0.653	0.252±0.022	-41.5
A4	Day1	123.4±4.101	0.164±0.018	-41.0
	Day30	359.2±4.413	0.304±0.041	-42.0
	Day60	361.1±5.413	0.378±0.022	-47.4
	Day90	372.9±22.14	0.411±0.038	-41.9
A5	Day1	172.5±1.733	0.139±0.025	-36.3

Table 4.2 (continued)

	Day30	342.3±6.123	0.293±1.578	-33.2
	Day60	401.5±3.421	0.398±2.115	-30.2
	Day90	678.3±2.113	0.767±2.523	-30.5
A6	Day1	159.1±3.168	0.214±0.081	-46.1
	Day30	172.4±0.722	0.174±0.010	46.5
	Day60	193.1±2.302	0.129±0.033	-42.3
	Day90	203.9±3.309	0.158±0.008	-40.1
A7	Day1	150.0±2.167	0.182±0.001	-51.2
	Day30	403.5±1.439	0.607±0.037	-50.3
	Day60	571.2±121.3	0.606±0.043	-43.2
	Day90	577.5±45.99	0.594±0.027	-40.7
A8	Day1	125.6±4.688	0.164±0.030	-37.4
	Day30	289.8±4.963	0.205±0.020	-38.5
	Day60	386.3±10.21	0.512±0.058	-47.3
	Day90	406.6±3.430	0.443±0.055	-37.4
A9	Day1	138.4±1.627	0.207±0.032	-34.3
	Day30	158.7±3.767	0.200±0.014	-33.5
	Day60	162.8±1.378	0.194±0.007	-43.4
	Day90	169.1±0.896	0.151±0.011	-33.0
B1	Day1	159.1±3.705	0.188±0.051	-42.5
	Day30	154.2±0.346	0.197±0.008	-43.9

Table 4.2 (continued)

	Day60	158.2±2.383	0.213±0.074	-40.3
	Day90	160.5±1.228	0.167±0.020	-36.2
B2	Day1	150.3±2.421	0.139±0.022	-42.4
	Day30	179.6±7.247	0.289±0.015	-40.8
	Day60	165.0±0.816	0.137±0.007	-41.2
	Day90	185.3±3.297	0.168±0.001	-42.0
B3	Day1	215.2±1.598	0.319±0.012	-37.0
	Day30	141.8±1.005	0.160±0.012	-36.2
	Day60	140.0±1.912	0.171±0.011	-37.1
	Day90	169.9±3.584	0.233±0.013	-36.0
B4	Day1	198.8±2.315	0.367±0.017	-37.0
	Day30	151.6±1.045	0.179±0.009	-36.0
	Day60	155.2±0.395	0.188±0.014	-36.8
	Day90	199.1±2.727	0.204±0.011	-39.0
B5	Day1	159.5±2.837	0.244±0.059	-43.2
	Day30	146.2±2.198	0.200±0.037	-40.8
	Day60	140.2±2.321	0.159±0.015	-44.8
	Day90	134.3±2.969	0.181±0.017	-38.0
B6	Day1	150.2±1.180	0.126±0.015	-33.5
	Day30	149.1±1.252	0.152±0.012	-31.7
	Day60	152.7±2.271	0.168±0.028	-42.2

Table 4.2 (continued)

	Day90	150.4±2.742	0.134±0.012	-37.4
C1	Day1	1429.0±135.2	0.457±0.014	-21.7
	Day30	1129.0±339.2	0.891±0.118	-33.0
	Day60	1234.0±138.1	0.858±0.107	-22.5
	Day90	1208.0±123.7	0.837±0.163	-28.3
C2	Day1	990.4±41.39	0.645±0.035	-25.1
	Day30	3078.0±97.79	0.937±0.055	-23.9
	Day60	2760.0±26.53	0.348±0.063	-18.6
	Day90	1416.0±84.00	0.715±0.034	-27.5
C3	Day1	1552.0±43.86	0.832±0.058	-25.7
	Day30	1190.0±144.7	0.802±0.093	-26.4
	Day60	881.7±6.942	0.701±0.115	-22.3
	Day90	807.8±152.4	0.719±0.185	-19.1
C4	Day1	1812.0±168.5	0.693±0.091	-25.5
	Day30	2104.0±167.6	0.784±0.131	-25.9
	Day60	2316.0±133.2	0.917±0.037	-20.8
	Day90	2899.0±107.2	0.848±0.035	-21.4
C5	Day1	1766.0±78.17	0.598±0.193	-26.2
	Day30	891.4±55.68	0.598±0.193	-24.5
	Day60	848.6±38.74	0.512±0.044	-23.8
	Day90	833.4±152.4	0.584±0.126	-25.5

Table 4.2 (continued)

C6	Day1	446.7±10.07	0.529±0.039	-25.4
	Day30	1356.0±286.8	0975±0.042	-26.1
	Day60	1409.0±13.99	0.839±0.061	-14.5
	Day90	1225.0±121.7	0.762±0.070	-27.5

Figure 4.4 The physical appearance of selected unloaded NLC dispersion; A1, B2 and B6

4.4 Formulation, characterization and stability of marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

According to the earlier study of unloaded NLC, formulation A1, B2 and B6 which showed good particle size stability, narrow size distribution and zeta potential were selected to develop for ME-NLC. From the previous result of antioxidant activity of H, EA, Et and 14 fractions of EA, EA and F9 were chosen to incorporate

into NLC as active ingredient because of their high antioxidant activity among all extracts. The amounts of EA and F9 in NLC was calculated based on IC_{50} from TBARS assay (91.48µg/ml and 38.95µg/ml, respectively)

EA-NLC was first developed following by F9-NLC based on the results from EA-NLC development. For EA-NLC, the concentration of surfactant (combining of Tween® and Span®) was varied from 5, 7, 10, to 12% to investigate the effect of surfactant's concentration. All of EA-NLC dispersions have yellow color, good smell, smooth and homogenous texture with low viscosity. Table 4.3 shows the results of particle size, PDI and zeta potential value of ME-NLC dispersion after 1, 30, 60 and 90 days of storage at room temperature. The incorporation of EA into NLC resulted in the larger particle size compared with unloaded-NLC while PDI and zeta potential did not effect by ME. But the particle size of EA-NLC tended to be larger during storage for 90 days this could be indicate that the aggregation of particles might occurred. Especially for EA-NLC from A1 formulation, the particle size performed at 30, 60 and 90 days after preparation were greatly increased and small amount of large particles was detected. However the particle size of all EA-NLC from B2 and B6 did not exceed 300nm remained in nanosize. For physical appearance and physical stability, after 30 days of storage, EA-NLC from A1 with 5, 12% surfactant, B2 and B6 with 5% surfactant were observed the creaming in dispersion. The small particles were optically observed in EA-NLC from B6 with 7% surfactant. Some part of particles seem to assemble like brownish curd as observed in EA-NLC from A1 with 5, 7, 10% surfactant, B2 with 5, 7, 10, 12% surfactant, B6 with 5, 10%surfactant. For B6 with 12%surfactant, it's remained the same as freshly prepared dispersion. The brownish curd might be the mixture of EA and PEG400, the solvent of extract, that

could be occur during the homogenization. EA might partition from melted lipid phase to the water phase and formed this curd.

At this point, EA-NLC with particle size larger than 500nm and/or unstable were excluded. Thus, B2 with 10, 12 %surfactant and B6 with 12%surfactant seemed to be suitable for incorporation of EA even though the smaller curd was observed in B2 with 10, 12 %surfactant as mentioned above. To solve this problem of B2, the concentration of PEG400 was increased from 4 to 6% in order to remain the extract in lipid phase. Since in general, the solubility of drugs in liquid lipid is higher than solid lipid [54]. After increasing of PEG400 concentration, the physical appearance after storage for 90 days of only B2 with 12%surfactant was improved.

All of EA-NLC were subjected to the stability test at three conditions; 4°C, room temperature and H/C cycling for 6 cycles. The results revealed (data not shown) that, at RT, EA-NLC from B2 with 12%surfactant-6%PEG400 were more stable than other formulations. No creaming, separation or aggregation was found. This formulation also showed good appearance as day 1 with slightly increasing of the viscosity at 4°C.

As mentioned above, F9-NLC was developed right after the development of EA-NLC was completed. B2 and B6 with 12% surfactant were selected to formulate F9-NLC. Due to the amount of F9 in formulation is less than EA thus 4%PEG400 should be adequate to dissolve F9. The obtained F9-NLC had pale yellow color, good smell, smooth and homogenous texture. The particle sizes of F9-NLC from B2 and B6 with 12% surfactant after storage for 90 days were lower than 200nm. (160.2 and 138.8nm, respectively). Both of them were stable after stability test at 4°C and RT conditions except H/C condition.

Table 4.3 The mean particle size, polydispersity index (PDI) and zeta potential value of marigold flower extract loaded NLC dispersion (ME-NLC) after storage for 30, 60 and 90 days.

Formulation		Particle size (nm)	PDI	Zeta potential (mV)
A1 –S(5)-EA	Day1	199.8±1.986	0.176±0.008	-42.8
	Day30	429.9±34.11	0.662±0.031	-38.0
	Day60	482.7±83.51	0.658±0.026	-46.9
	Day90	-	-	-
A1-S(7)-EA	Day1	387.5±6.448	0.266±0.022	-32.1
	Day30	969.8±88.50	0.758±0.023	-42.2
	Day60	978.3±25.08	0.755±0.064	-48.1
	Day90	1070.0±353.1	0.921±0.137	-39.9
A1-S(10)-EA	Day1	244.4±3.859	0.210±0.028	-34.6
	Day30	672.6±39.22	0.735±0.029	-48.8
	Day60	529.7±108.0	0.606±0.012	-47.9
	Day90	1121.0±286.2	0.885±0.105	-50.0
A1-S(12)-EA	Day1	173.3±3.623	0.195±0.004	-41.9
	Day30	549.8±41.74	0.678±0.145	-47.4
	Day60	568.6±68.19	0.713±0.022	-44.6

Table 4.3 (continued)

	Day90	-	-	-
B2-S(5)-EA	Day1	175.3±3.705	0.223±0.030	-35.1
	Day30	237.8±0.946	0.258±0.013	-33.3
	Day60	282.1±10.87	0.347±0.016	-43.1
	Day90	-	-	-
B2-S(7)-EA	Day1	151.0±2.61	0.189±0.16	-34.5
	Day30	161.9±1.301	0.225±0.004	-42.1
	Day60	176.3±10.61	0.294±0.067	-45.0
	Day90	265.0±19.67	0.464±0.016	-45.1
B2-S(10)-EA	Day1	157.3±1.735	0.167±0.016	-40.3
	Day30	156.4±1.629	0.164±0.011	-45.1
	Day60	156.8±0.451	0.183±0.009	-50.3
	Day90	171.5±15.86	0.196±0.013	-49.5
B2-S(12)-EA	Day1	146.2±1.246	0.139±0.009	-48.8
	Day30	156.3±2.668	0.141±0.015	-39.0
	Day60	153.2±1.881	0.106±0.007	-41.7
	Day90	182.1±0.767	0.156±0.032	-42.3
B6-S(5)-EA	Day1	153.6±1.589	0.129±0.013	-42.9
	Day30	157.5±2.421	0.176±0.016	-39.0
	Day60	154.0±2.315	0.202±0.019	-46.3
	Day90	-	-	-

Table 4.3 (continued)

B6-S(7)-EA	Day1	144.5±3.968	0.122±0.019	-34.9
	Day30	146.2±0.500	0.120±0.018	-41.9
	Day60	148.1±2.776	0.178±0.006	-50.1
	Day90	299.4±8.621	0.437±0.014	-46.0
B6-S(10)-EA	Day1	146.8±0.825	0.115±0.004	-36.3
	Day30	143.4±0.972	0.106±0.001	-43.6
	Day60	162.3±2.661	0.751±0.018	-49.5
	Day90	192.7±2.564	0.283±0.014	-47.5
B6-S(12)-EA	Day1	136.0±0.090	0.117±0.020	-49.0
	Day30	140.2±0.324	0.069±0.004	-42.4
	Day60	176.5±0.740	0.091±0.011	-42.7
	Day90	191.0±0.287	0.079±0.020	-42.2
B2-S(10)-PEG400(6)-EA	Day1	142.9±1.299	0.159±0.019	-36.0
	Day30	157.4±2.600	0.197±0.034	-49.3
	Day60	159.4±2.395	0.204±0.022	-39.0
	Day90	198.2±9.341	0.328±0.036	-42.3
B2-S(12)-PEG400(6)-EA	Day1	126.3±1.472	0.131±0.016	-43.8
	Day30	159.5±2.518	0.205±0.010	-41.1
	Day60	177.8±1.017	0.149±0.020	-36.2
	Day90	220.1±1.971	0.205±0.017	-38.4
B2-S(12)-PEG400(4)-F9	Day1	146.9±0.617	0.140±0.008	-38.1

Table 4.3 (continued)

	Day30	154.7±2.695	0.187±0.031	-36.3
	Day60	153.9±3.309	0.197±0.022	-40.8
	Day90	160.2±2.353	0.222±0.015	-46.1
B6-S(12)-PEG400(4)-F9	Day1	149.8±0.981	0216±0.011	-41.7
	Day30	140.4±1.056	0.140±0.019	-40.2
	Day60	141.1±1.001	0.177±0.030	-37.7
	Day90	138.8±1.024	0.166±0.004	-41.7±

S=surfactant

4.5 Selection of good marigold flower extract loaded nanostructured lipid carriers (ME-NLC)

By combining results on physical appearance stability and stability test, the results suggested that good physical stability and good dispersion quality of ME-NLC could be obtained from B2 with 12%surfactant-6%PEG400 for EA, B2 and B6 with 12% surfactant-4%PEG400 for F9. But for F9, B2 was more suitable for incorporating into cosmetic cream than B6 because of it showed higher viscosity. In conclusion, B2 with 12%surfactant-6%PEG400 and B2 with 12%surfactant-4%PEG400 formulations were chosen for preparation of EA-NLC and F9-NLC, respectively.

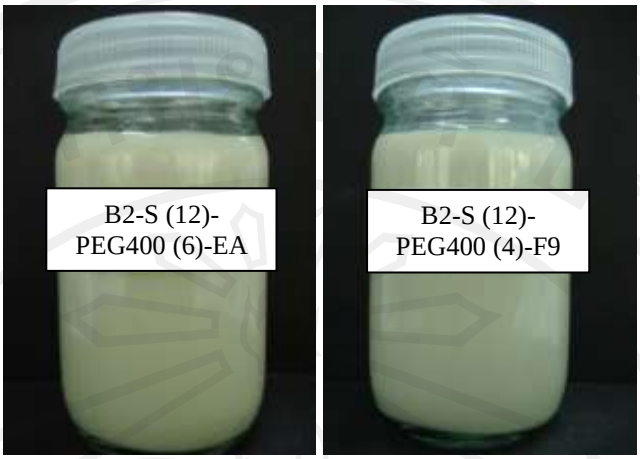


Figure 4.5 The physical appearance of selected EA-NLC and F9-NLC

4.6 Formulation, stability and selection of good cream base

All 6 freshly prepared cream bases had good appearances with white color, smooth and homogenous texture. The pH of all cream base formulations were in range of 5.0-6.0 and after storage in H/C cycling for 6 cycles, they showed nearly no difference from freshly prepared cream. Formula I, II, III and IV were not stable after 6 cycles of H/C cycling. Formula I, II and III had rancid odor. The separating of fluid was observed in formula I and II while creaming was observed in formula III and IV. Only Formula V was stable after stability test. Therefore, formula V was suitable for developing of loaded-NLC cream.

For formula VI, it was developed later after stability and antioxidant activity test of loaded-NLC cream prepared by mix the loaded NLC directly to selected cream base (formula V). After H/C cycling, the viscosity of loaded-NLC cream was lower than freshly prepared cream which was not suitable for cosmetic purpose. Then formula VI was developed by adding of Carbopol as thickening agent into the

formulation to improve the viscosity of cream base. Freshly prepared cream of formulation VI showed higher viscosity than the others. After stability test, the viscosity of formula VI did not changed and pH was 5.0. Thus, formula VI was chosen for further preparation of loaded-NLC cream.

4.7 Formulation and antioxidant activity of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

Conventional NLC dispersion contain about 10-20% (w/w) of lipid matrix and 80-90% of water. As a result, NLC dispersions possess a low viscosity. Therefore, NLC dispersions usually have to be incorporated in a convenient topical dosage form like creams or hydrogel to obtain a topical application form having the desired semisolid consistency. [1, 54]

In this study, NLC containing creams were prepared by directly mixing of cream base and selected NLC dispersions with a gentle stirring. Each ME-NLC was prepared at two concentrations of ME-NLC in cream which are 15 and 30%. The obtained ME creams and ME-NLC containing cream had light yellow color by EA was yellowish than F9, smooth and homogenous consistency. All of NLC containing creams; 15%EA-NLC cream, 30%EA-NLC cream, 15%F9-NLC cream, 30%F9-NLC cream, were investigated for physical stability and antioxidant activity stability compared with cream base and ME creams which have an identical final concentration of ME in formulations; 0.0225%EA cream, 0.045%EA cream, 0.009%F9 cream and 0.018%F9 cream, respectively. Overall tested creams were nine formulations.

At the first stage, NLC dispersions were incorporated into cream base formula V resulting in the low viscosity of formulations after stability test. So cream base formula VI was developed by adding the thickening agent; Carbopol, in the formulation and gave desirable viscosity for cosmetic application to the formulation.

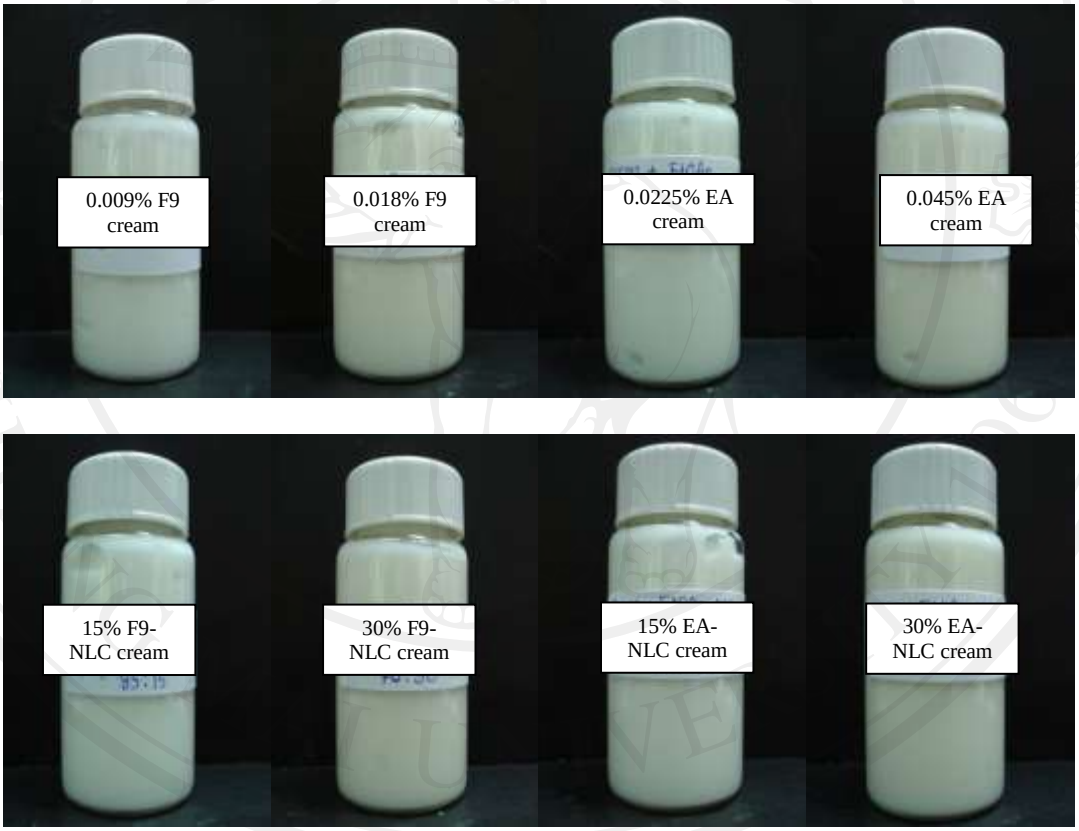


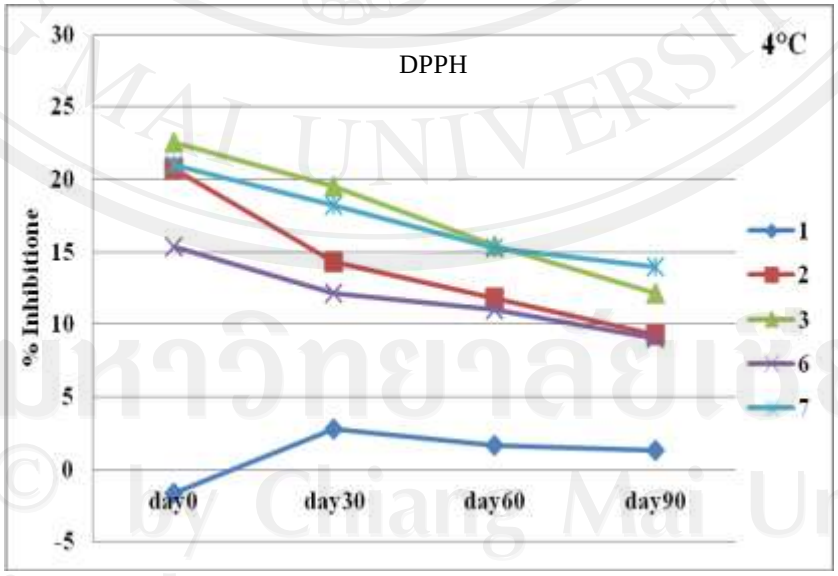
Figure 4.6 The 8 formulations of marigold cream; 15%EA-NLC cream, 30%EA-NLC cream, 15%F9-NLC cream, 30%F9-NLC cream, 0.0225%EA cream, 0.045%EA cream, 0.009%F9 cream and 0.018%F9 cream

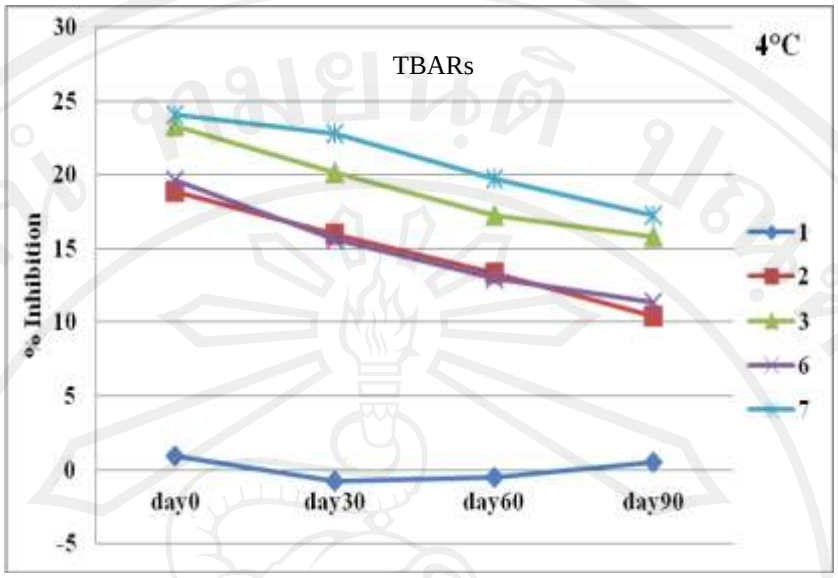
To consider for F9 containing formulation, after storage at 5 conditions; 4°C, 45°C, RT with light and without light and H/C cycling, the result revealed at RT with light, the color of all tested creams changed to pale yellow. The viscosity of F9 -NLC creams was lower than F9 cream at all conditions. The particle size and zeta potential were determined only F9-NLC creams. As tested creams were not stable at 45°C and H/C, the measurements could not perform. The obtained results demonstrated that, the particle sizes of nanoparticles were increased during storage time. At day 90, the particles sizes of 15% and 30%F9-NLC cream were in range 311.4 to 566.9nm. The increasing of particle sizes indicated that the aggregations of particles might be occurred during storage which was related with the decreasing of zeta potential values. Less zeta potential values increase the chance of particles aggregation in NLC. However, observed zeta potential values were > $|30|$mV suggested that NLC possessed good stability during storage. For antioxidant activity of F9-NLC containing cream compared with those F9 cream were performed at Day 0, 30, 60 and 90 of storage at 5 conditions. Antioxidant activities of all tested creams were determined by DPPH and TBARS assay. The results, reported in term of percent inhibition, were shown in Figure 4.7. The decreasing of percent inhibition during storage time at all conditions was observed in all tested creams. At 4°C Day 90, 30%F9-NLC cream showed the highest percent inhibition among all F9 tested creams and significantly different from 0.018%F9 cream from both DPPH and TBARS assay. At RT with light Day 90, the percent inhibition by TBARS assay of F9-NLC creams were higher than F9 creams with significantly difference. This might say that the incorporation of F9 into NLC can increase the stability of F9 to light resulting in the

higher percent inhibition. From this result, 30%F9-NLC cream was selected for further wrinkle reducing capacity test.

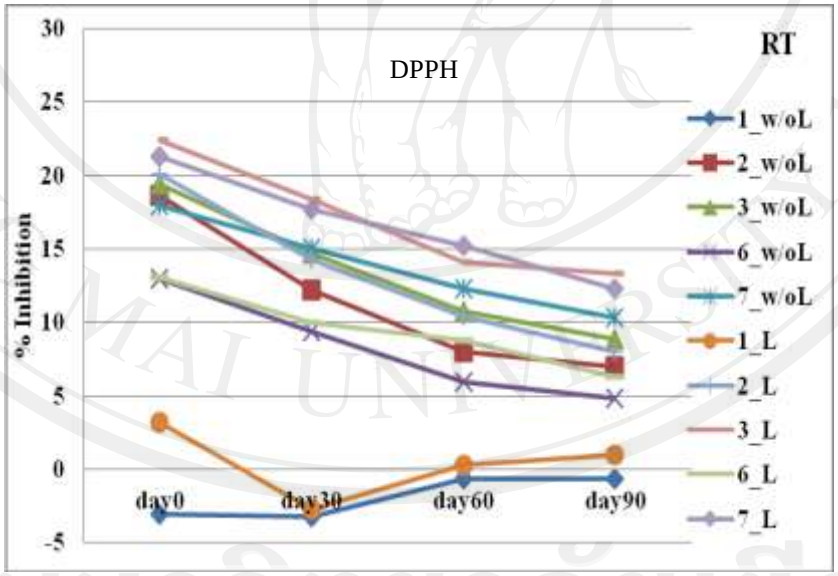
The formulations with EA as active compounds are 0.0225% and 0.045%EA cream, 15% and 30%EA-NLC cream. The physical appearance stability and particle size demonstrated the same results as F9 formulations. The particle size of EA-NLC creams after 90 days of storage at 5 conditions were in range 333.7-453.3 nm and zeta potential values were > 30mv. For antioxidant activity, the results were shown in Figure 4.8. At 4°C Day 90, the percent inhibition of 0.045%EA cream by DPPH assay showed significantly higher than 30%EA-NLC cream while RT with and without were not significantly difference. The percent inhibition by TBARS assay at 4°C and RT with and without light, 30%EA-NLC cream revealed the higher percent inhibition than 0.045%EA cream.

(a) 4°C





(b) room temperature with light and without light



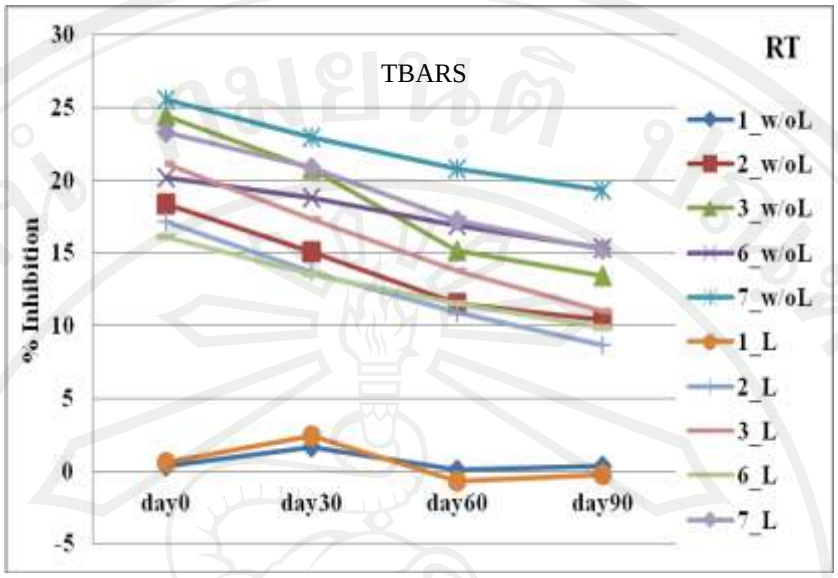
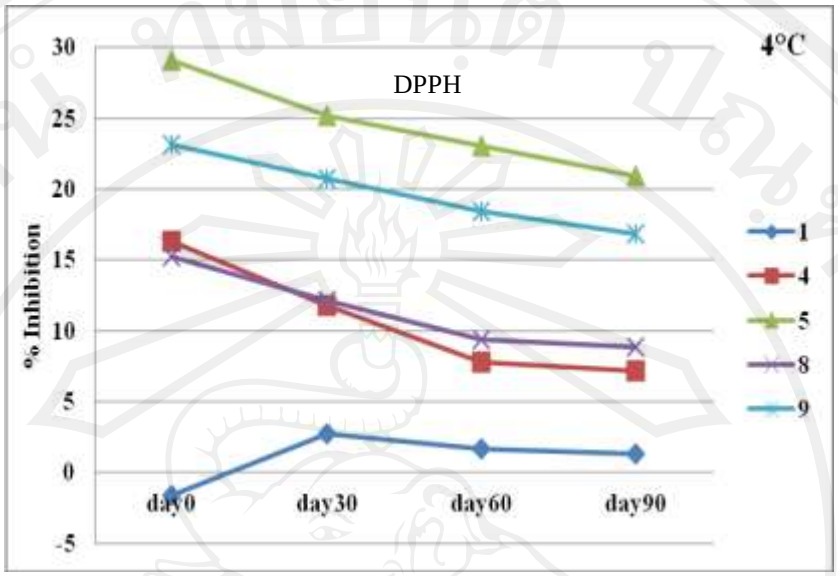
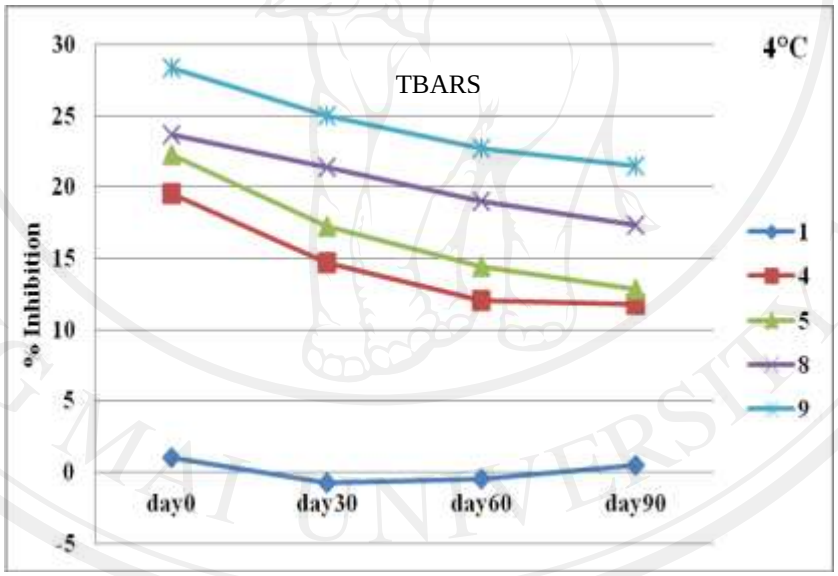


Figure 4.7 The antioxidant activity (percent inhibition) of F9 creams at 0, 30, 60 and 90 days after storage at different conditions (the results at 45°C and H/C cycling not shown) by DPPH and TBARS assay 1=cream base, 2=0.009%F9 cream, 3=0.018%F9 cream, 6=15%F9-NLCcream, 7=30%F9-NLC cream

To consider at 45°C by DPPH assay, the percent inhibition of 30%EA-NLC cream was significantly higher than 0.045%EA and 0.0225%EA cream was higher than 15%EA-NLC cream with no significance. While TBARS assay, the percent inhibition of 30%EA-NLC containing cream was higher than 0.045%EA with no significance and 0.0225%F9 cream was significance higher than 15%EA-NLC containing cream. These results may suggest that the incorporation of EA into NLC can partly increase the stability of EA to light.

(a) 4°C





(b) room temperature with light and without light

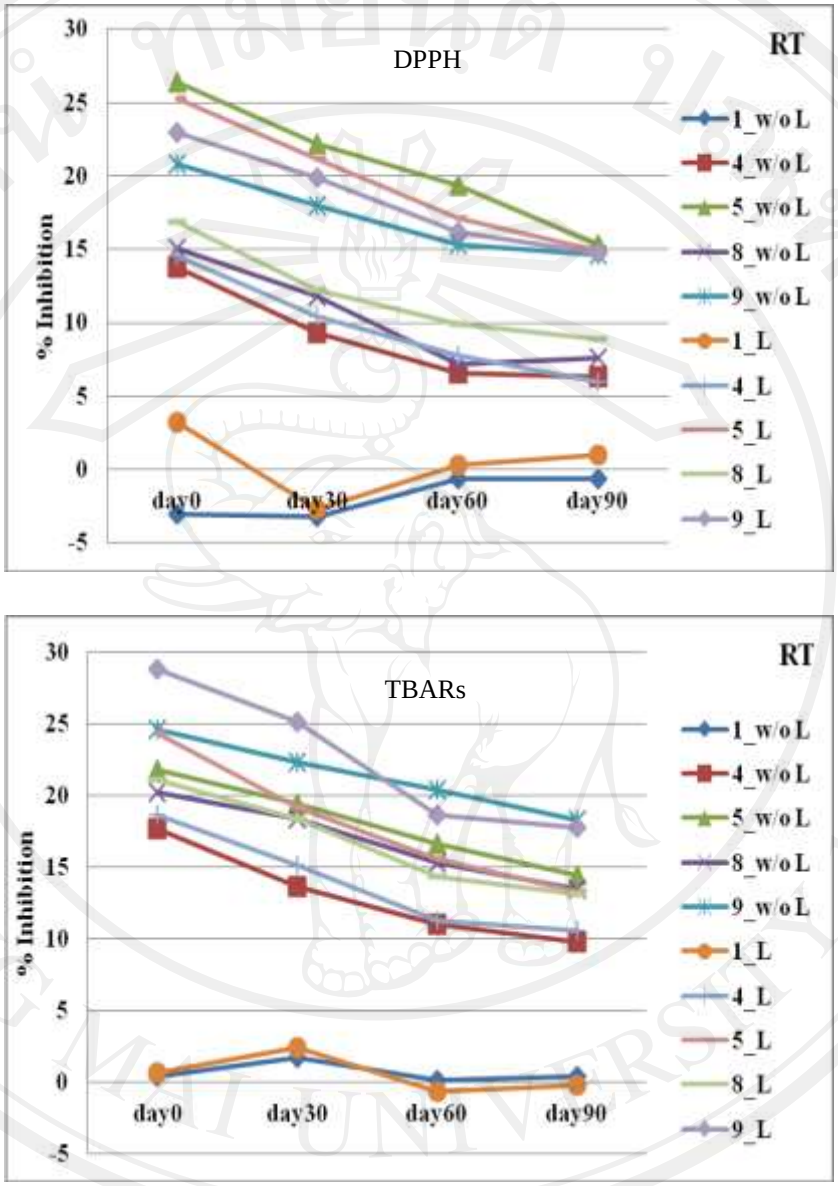


Figure 4.8 The antioxidant activity (percent inhibition) of EA creams at 0, 30, 60 and 90 days after storage at different conditions (the results at 45°C and H/C cycling not shown) by DPPH and TBARS assay 1 =cream base, cream, 4=0.0225%EA cream, 5= 0.045%EA cream, 8=15%EA-NLC cream and 9=30%EA-NLC cream

4.8 Selection of good marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

By combining results on physical appearance stability, stability test and antioxidant activity, the results suggested that good physical stability and good antioxidant activity of ME-NLC cream which suitable for cosmetic purpose could be obtained from 30%F9- cream and 30%EA-NLC cream.

4.9 Skin irritation testing in animal

The Draize model and its modification are commonly used to assay skin irritation using albino rabbits. The ME extracts (EA and F9), unloaded NLC, ME-NLC, cream base and ME-NLC cream were assessed of skin irritation by modified Draize Rabbit Models. The values of primary irritation index of these test substances were shown in Table 4.4. All of them exhibited no irritation on the rabbits' skin with PII values < 2.

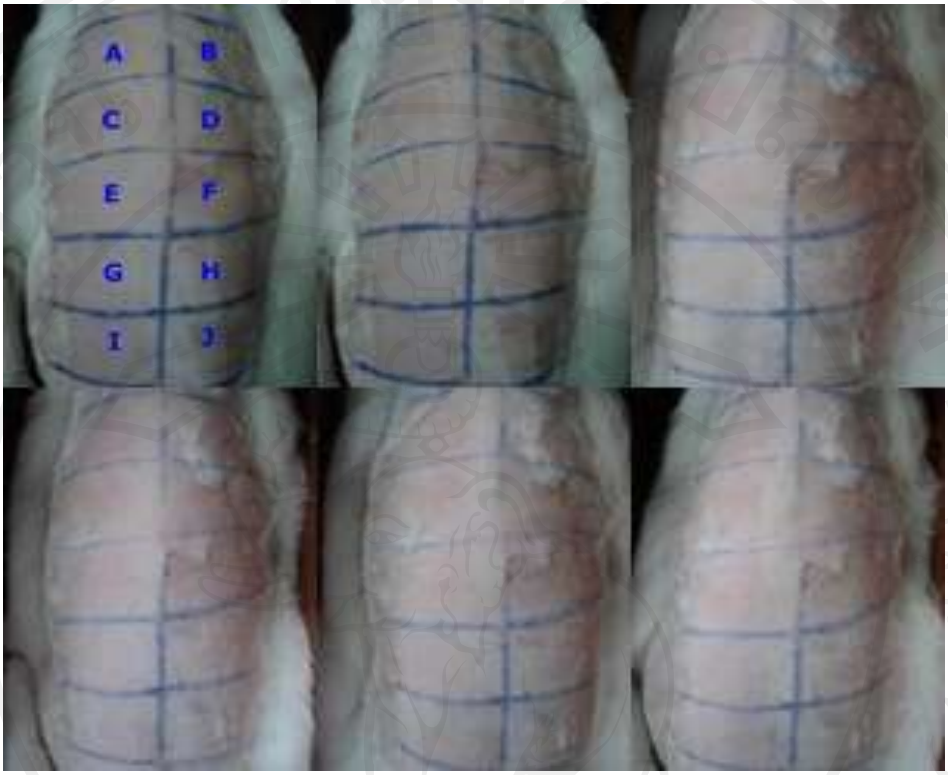
4.10 Skin irritation testing in human volunteers

The Finn Chamber® occlusive patch test was used to study skin irritation in human. In human skin irritation study, twenty five volunteers were test with 8 substances. The sequence of application was shown in Figure 4.10. According to the result in Table 4.5, most of test substances exhibited no irritation (PII<2) whereas 1.5%w/v sodium lauryl sulfate (SLS) as positive control revealed moderately irritating (PII=3.83).

Table 4.4 Primary irritation index (PII) and skin irritation reaction in rabbits

Test subsatances	PII	Classification of skin irritation
EA extract	0	Non irritating
F9 extract	0	Non irritating
Unloaded A1	0	Non irritating
0.15%EA A1	0	Non irritating
Unloaded B2	0	Non irritating
0.15%EA B2	0.25	Non irritating
Unloaded B6	0	Non irritating
0.15%EA B6	0	Non irritating
Distilled water	0	Non irritating
Cream base	0	Non irritating
0.025% EA Cream	0	Non irritating
0.045%EA Cream	0	Non irritating
0.009%F9 Cream	0	Non irritating
0.018%F9 Cream	0	Non irritating
30% EA -NLC Cream	0	Non irritating
15% EA -NLC Cream	0	Non irritating
30%F9-NLC Cream	0	Non irritating
15% F9-NLC Cream	0	Non irritating

Before 0 hr 1hr



24 hr 48 hr 72hr

Figure 4.9 Example of skin irritation test in rabbit of 10 substances; A= distilled water, B= Cream base, C= %0.009F9 Cream, D= 0.018%F9 Cream, E= 0.025%EA Cream, F= 0.045%EA Cream, G= 15%F9-NLC Cream, H= 30%F9-NLC Cream, I= 15%EA -NLC Cream, J= 30%EA -NLC Cream

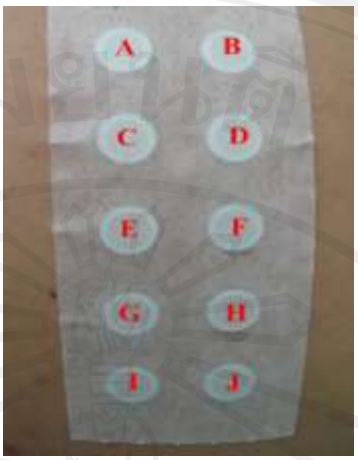


Figure 4.10 Sequence of application on skin irritation testing in human A = EA -NLC Cream, B = F9-NLC Cream, C = EA Cream, D = F9 Cream, E = EA extract, F = F9 extract, G = cream base, H = fraction 9.7 extract, I = untreated site and J = 1.5% sodium lauryl sulfate as positive control.

Table 4.5 Primary irritation index (PII) and skin irritation reaction in 25 volunteers of marigold flower extracts and selected creams

Test subsatances	PII	Classification of skin irritation
1.5%w/v Sodium lauryl sulfate	3.83	Moderately irritating
EA extract	0	Non irritating
F9 extract	0	Non irritating
Cream base	0.17	Non irritating
EA Cream	0	Non irritating
F9 Cream	0	Non irritating
EA -NLC Cream	0.17	Non irritating
F9-NLC Cream	0	Non irritating

Figure 4.11 The example of skin irritation test in human with Finn Chamber®; erythema reaction was observed at 3 sites (A= 30% EA-NLC Cream, G = cream base and J = 2% sodium lauryl sulfate in volunteer no.10.

4.11 Wrinkle reducing capacity of marigold flower extract loaded nanostructured lipid carriers cream (ME-NLC cream)

The assessment of wrinkle reducing capacity of ME-NLC cream by Skin-visiometer® SV 600 FW resulted in four parameters (volume, surface, roughness R*a* and Rz). The values of four parameters of each treatment were compared between before and after application at $p < 0.05$. To compare the difference of all treatment, the percent efficiency were calculated and statically analyzed at $p < 0.05$. Figure 4.12 showed the mean value of four parameters of twenty five subjects. None of them discontinued this clinical study

According to the result in Table 4.6, 0.018%F9 cream, 0.045%EA cream, 30%F9-NLC cream , 30%EA-NLC cream sites showed the changes of all parameters with significantly reducing in volume, surface, R*a* and Rz except Rz value of

0.018%F9 cream and 0.045%EA cream showed no significantly change after 8 weeks of application. The untreated and placebo (cream base) sites presented significantly increasing on surface value (+0.66 and +0.41, respectively) while volume, R*a* and R*z* increased with no significance except R*z* value of placebo, it showed non-significantly decreasing. From these results we might stated that all creams containing marigold extracts have the capability to reduce the wrinkles of skin.

The differences among six treatments were analyzed by Duncan's multiple range test at $p<0.05$ after application for 8 weeks. As assessed to percent efficiency value, application of 30%F9-NLC Cream, 30% EA-NLC Cream showed significantly difference against untreated and placebo site for all parameters. Consider to the formulations contained the same marigold extracts (F9 and EA), 30%F9-NLC Cream exhibited significantly difference with 0.018%F9 cream in surface, R*a* and R*z* except volume parameter, it exhibited non-significantly difference. For 30% EA -NLC cream versus 0.045% EA cream, they exhibited non-significantly difference in all parameters. Between untreated and placebo site, they showed significantly difference in volume and surface while R*a* and R*z* did not.

Table 4.6 The wrinkle reducing capacity on the R*a*, R*z*, volume and surface after 4 and 8 weeks of treatment.

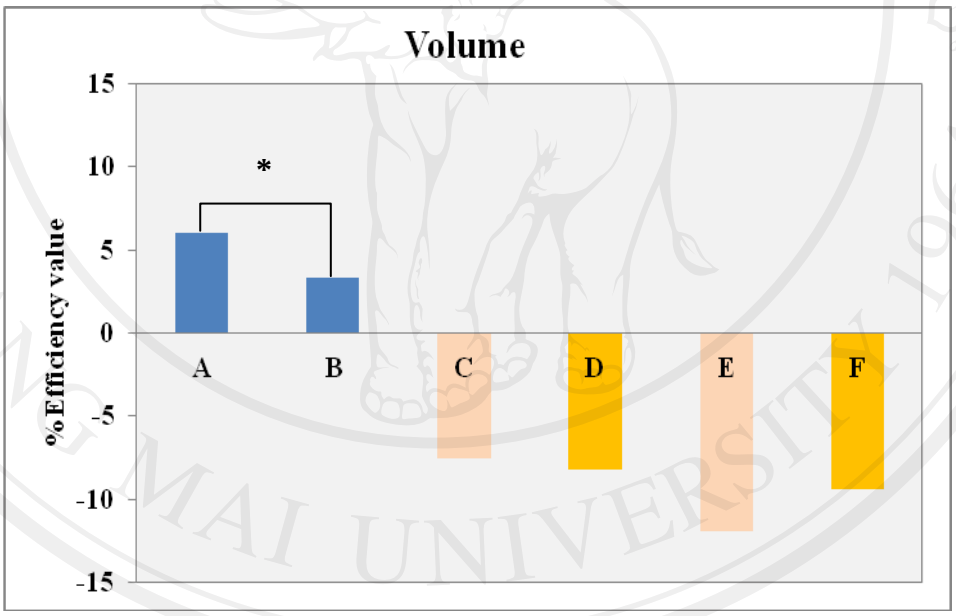
	Wrinkle reducing parameter			
	volume	**surface**	**roughness**	
			R*a*	**Rz**
Untreated area				
Day0	47.87 ± 8.89	4.59 ± 0.50	10.10 ± 1.98	41.34 ± 6.30
Day30	50.77 ± 7.39	5.06 ± 0.67	10.56 ± 1.87	42.83 ± 6.91
Day60	50.74 ± 8.28	5.25 ± 0.63*	10.90 ±1.85*	45.16 ± 5.96
cream base (placebo)				
Day0	48.73 ± 6.55	4.75 ± 0.46	10.24 ± 1.40	43.47 ± 5.52
Day30	49.31 ± 6.98	4.73 ± 0.49	10.36 ± 1.58	43.49 ± 5.93
Day60	50.53 ± 7.38	5.16 ± 0.50*	10.38 ± 1.64	42.97 ± 5.99
0.018%F9 Cream				
Day0	51.09 ± 8.23	4.83 ± 0.50	10.87 ± 1.73	46.58 ± 7.16
Day30	49.44 ± 6.77	4.70 ± 0.51	10.40 ± 1.42	46.39 ± 7.39
Day60	47.43 ± 7.06*	4.53 ± 0.50*	10.21 ± 1.56*	44.70 ± 6.62
0.045%EA Cream				
Day0	47.37 ± 7.23	4.99 ± 0.40	10.54 ± 1.53	46.04 ± 5.80
Day30	45.50 ± 6.56	4.85 ± 0.45	10.13 ± 1.28	43.90 ± 5.66
Day60	44.42 ± 6.20*	4.70 ± 0.57*	9.59 ± 1.58*	42.95 ± 5.97
30%F9- NLC Cream				
Day0	48.91 ± 8.52	4.68 ± 0.43	10.18 ± 1.71	42.28 ± 6.05
Day30	46.11 ± 7.31	4.42 ± 0.47	9.76 ± 1.60	39.86 ± 6.27
Day60	44.06 ± 8.13*	4.10 ± 0.63*	8.89 ± 1.60*	38.52 ± 5.75*

Table 4.6 (continued)

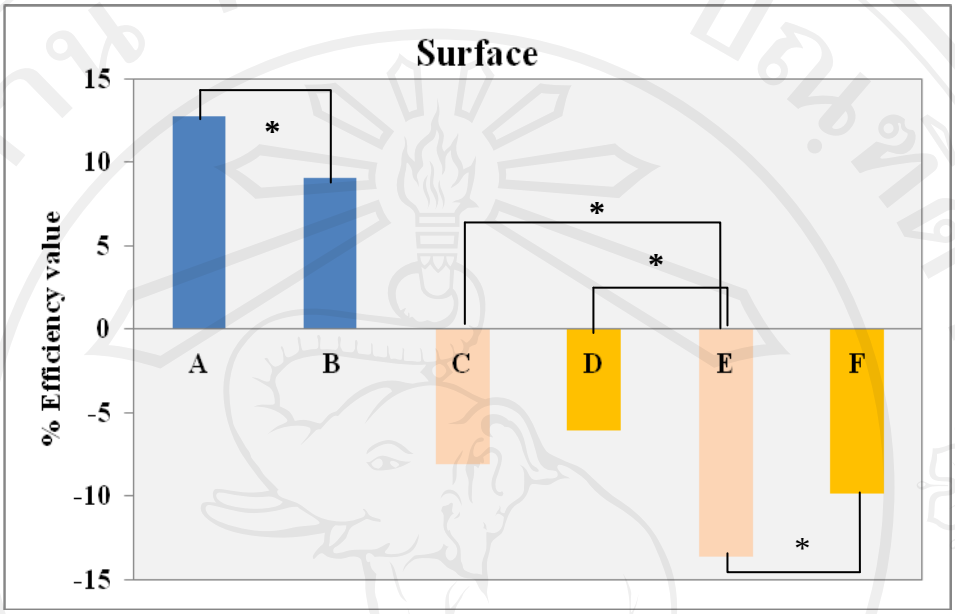
30%EA-NLC Cream				
Day0	48.80 ± 6.97	4.93 ± 0.47	10.70 ± 1.28	45.42 ± 5.65
Day30	46.92 ± 6.57	4.65 ± 0.58	10.10 ± 1.30	43.35 ± 5.82
Day60	44.43 ± 6.28*	4.48 ± 0.57*	9.38 ± 1.59*	41.85 ± 6.00*

* The mean difference is significant at the 0.05 level compared between initial value and 8 weeks after application.

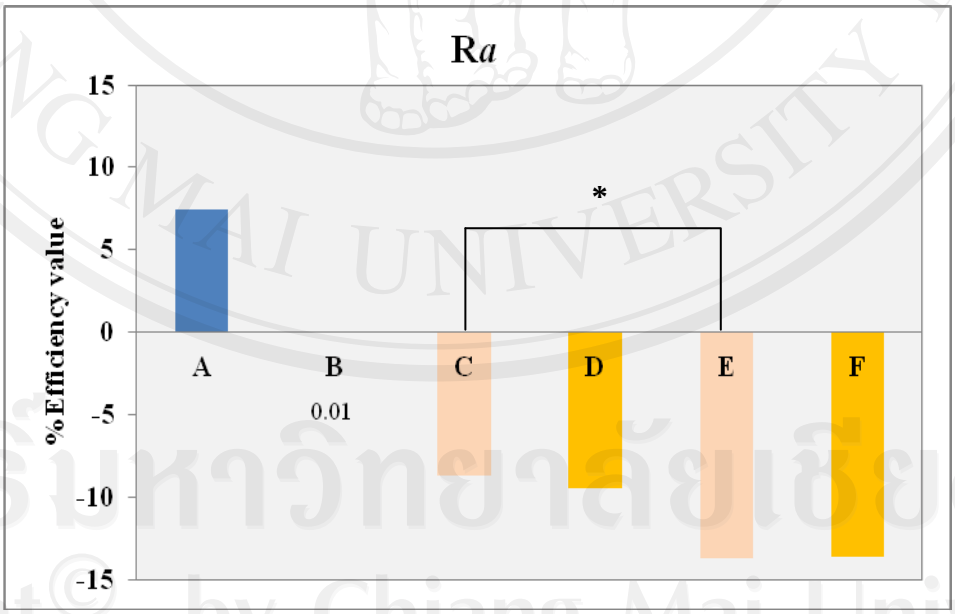
(a) Volume



(b) Surface



(*c*) R*a*



(c) Rz

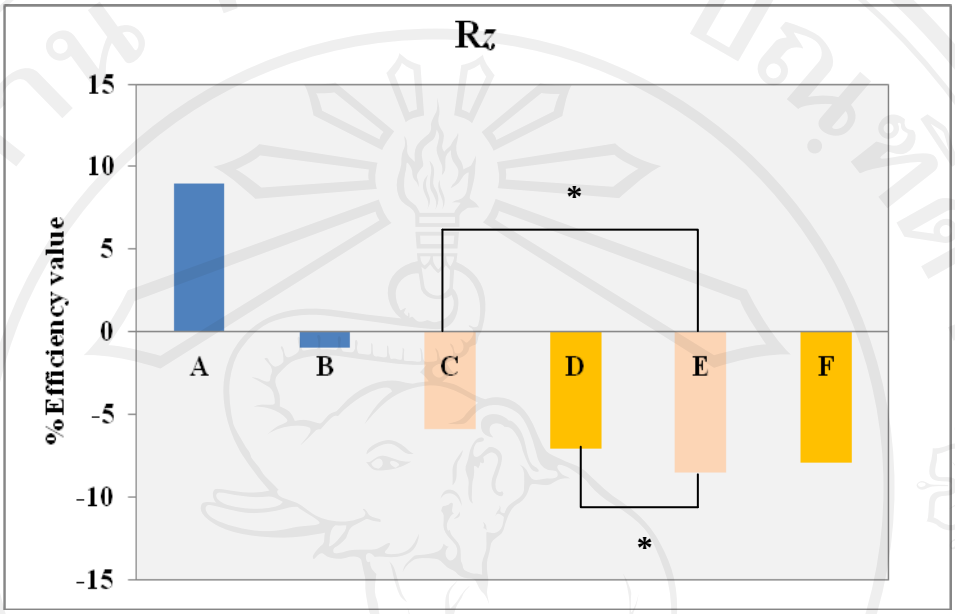
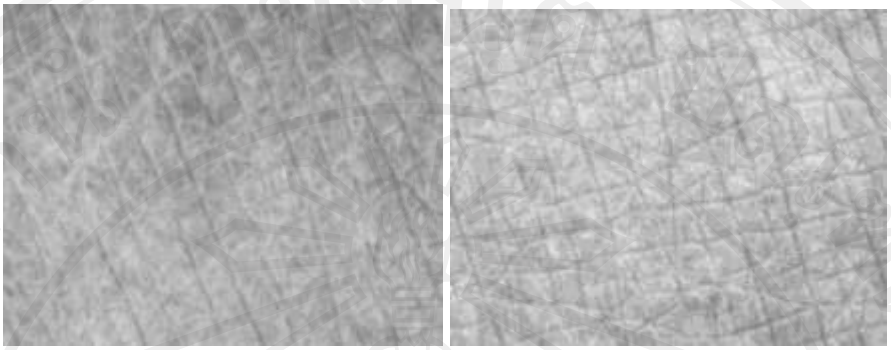


Figure 4.12 The percent efficiency value on volume, surface, R*a* and Rz parameter after 8 weeks of treatment at $p<0.05$; A=untreated area, B=placebo area, C= 0.018%F9 cream, D= 0.045%EA cream, E= 30%F9-NLC cream and F= 30%EA -NLC cream

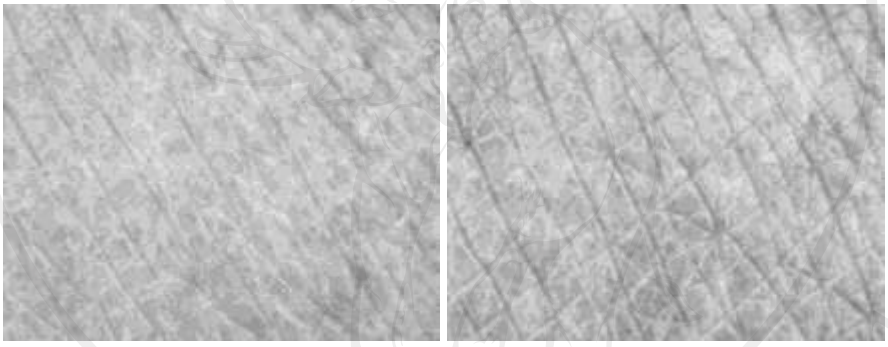
After 8 weeks of application, the depths of wrinkles were obviously reduced when comparing between before and after treatment in creams containing both ME and ME-NLC, especially in 30%F9-NLC and 30%EA-NLC cream, as shown in Figure 4.13. Where in untreated and cream base areas, the wrinkle reducing ability were not obviously found. It could be concluded that the antioxidant capacity of ME pays the important role in skin wrinkle reducing efficacy and the entrapment of ME into NLC also showed better effects.

(*a*) Untreated area



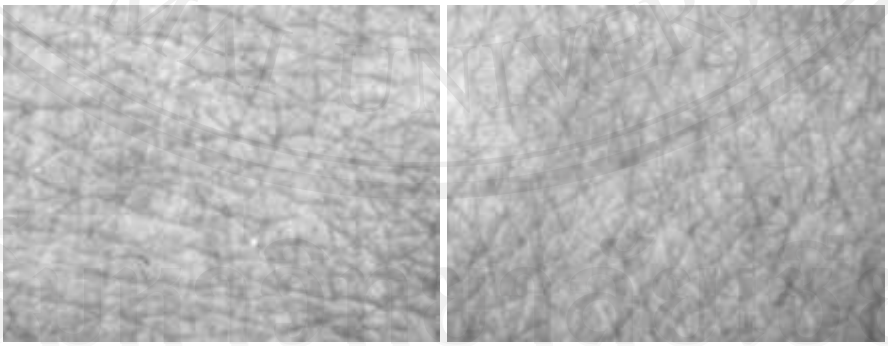
before after 8 weeks

(*b*) Placebo area



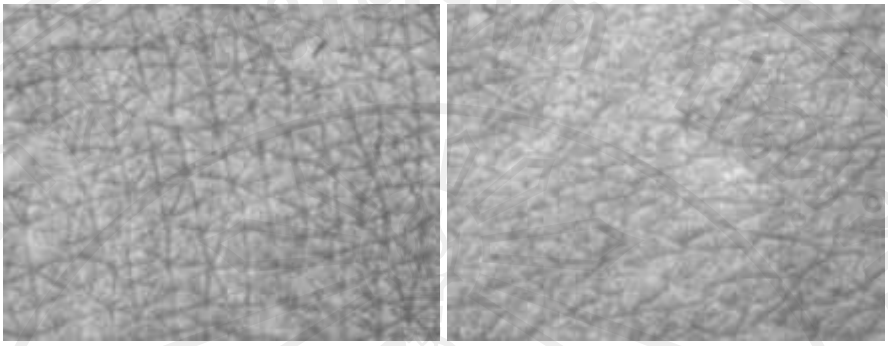
before after 8 weeks

(*c*) 0.018%F9 cream area



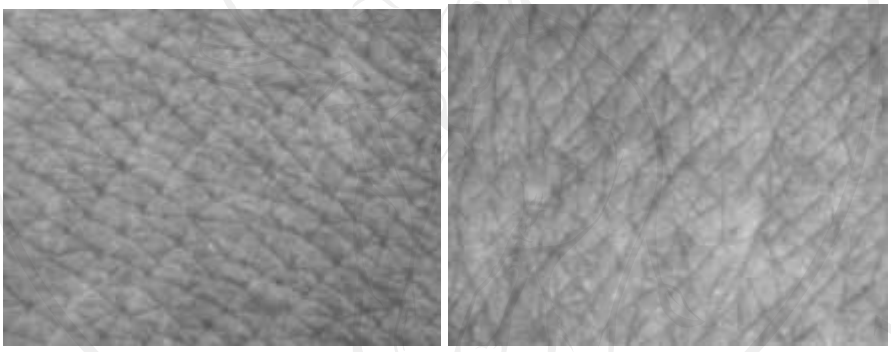
before after 8 weeks

(*d*) 0.045%EA cream area



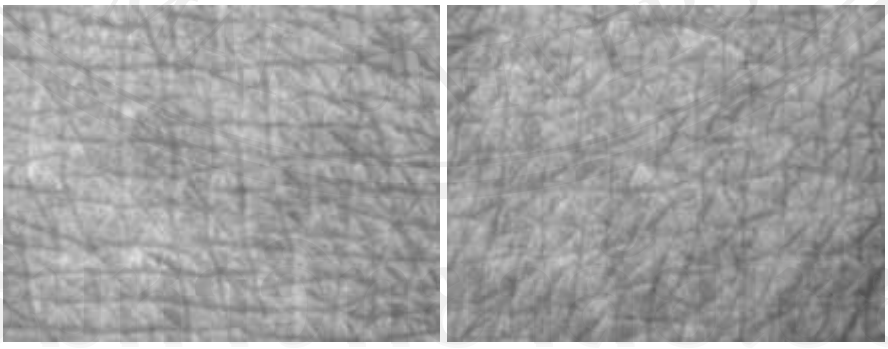
before after 8 weeks

(*e*) 30%F9-NLC cream



before after 8 weeks

(*f*) 30%EA-NLC cream



before after 8 weeks

Figure 4.13 Skin surfaces of forearms using Skin-Visiometer® SV 600 FW before and after 8 weeks of treatment

The level of subjects' satisfaction is usually assessed by asking subjects to rate how much they like the test creams overall, using a five-point Like scale. The results are shown in Table 4.6 - 4.7. The satisfaction results ranged from like extremely to medium like exhibited more than 80% for all topics; cream texture, color, spreadability, softness, greasiness, tackiness of cream and also overall satisfaction. While dislike extremely shown not more than 10% for all assessed topics. No subject was observed for skin irritation reaction during testing period. All of them were willing to use these products again if they had the chance.

Table 4.7 The percentage of satisfaction on test cream before and after use

Topic	The satisfaction before use (%)				
	Like extremely	Very much	Medium like	Like slightly	Dislike extremely
1.Cream texture					
cream base	28	36	32	4	0
F9 cream	28	24	44	4	4
EA cream	16	28	48	4	0
F9-NLC cream	28	40	32	0	0
EA -NLC cream	16	44	28	12	0
2.Color					
cream base	24	44	28	4	0
F9 cream	24	24	52	0	0

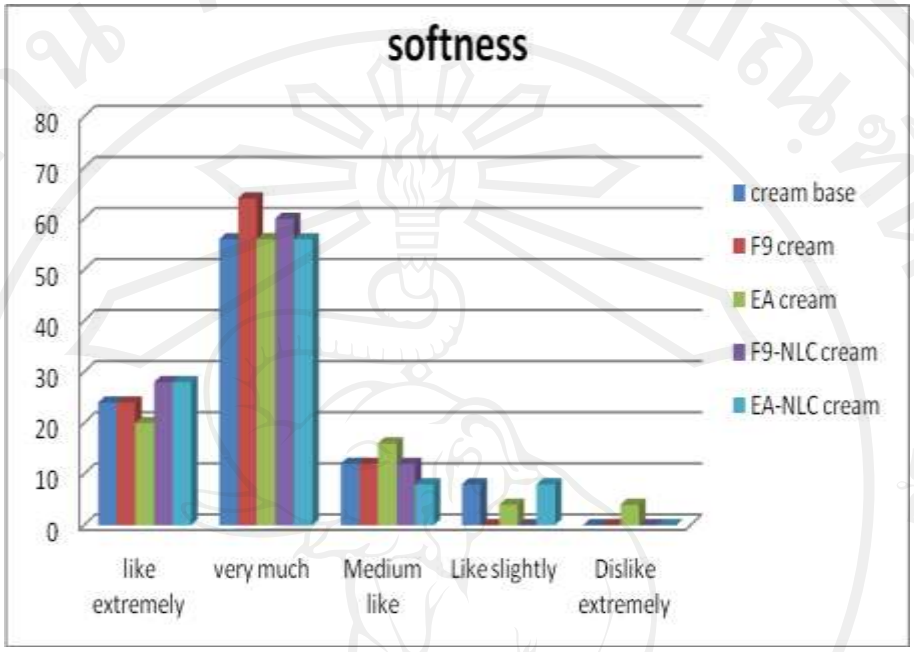
Table 4.7 (continued)

EA cream	20	36	36	4	4
F9-NLC cream	20	36	44	0	0
EA -NLC cream	12	40	36	12	0
Topic	The satisfaction after use (%)				
	Like extremely	Very much	Medium like	Like slightly	Dislike extremely
1.Softness of cream					
cream base	24	56	12	8	0
F9 cream	24	64	12	0	0
EA cream	20	56	16	4	4
F9-NLC cream	28	60	12	0	0
EA -NLC cream	28	56	8	8	0
2.Spreadability					
cream base	24	60	8	8	0
F9 cream	16	48	36	0	0
EA cream	8	56	24	8	4
F9-NLC cream	16	72	12	0	0
EA -NLC cream	20	60	12	8	0
3.Greasiness of cream					
cream base	12	24	40	20	0
F9 cream	16	36	40	4	4
EA cream	16	32	32	16	4

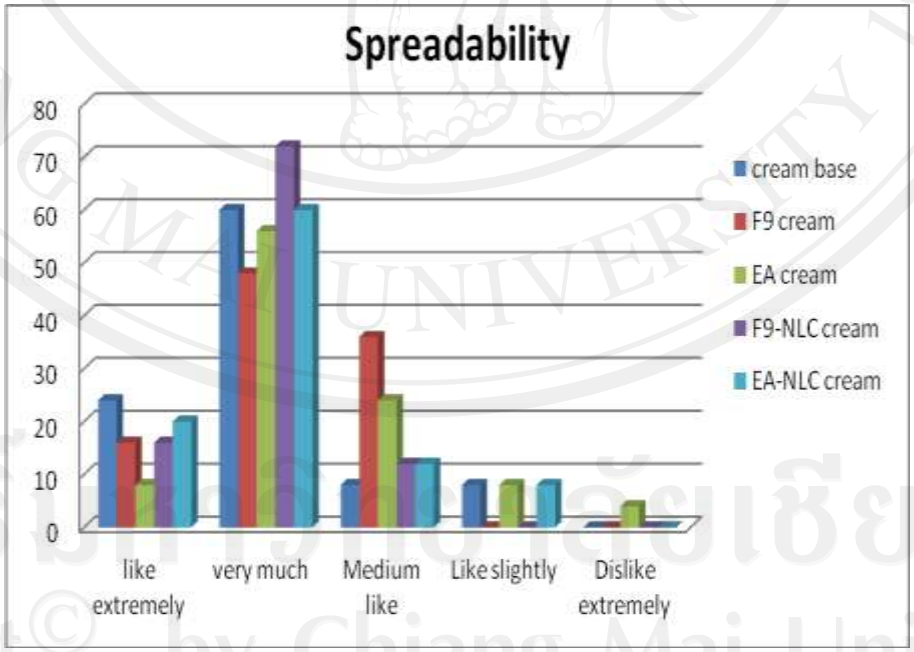
Table 4.7 (continued)

F9-NLC cream	4	44	32	12	0
EA -NLC cream	8	36	36	20	0
4.Tackiness of cream					
cream base	20	24	48	0	8
F9 cream	24	48	12	12	4
EA cream	24	40	32	0	4
F9-NLC cream	4	44	44	8	0
EA -NLC cream	4	56	36	4	0
5.Overall satisfaction					
cream base	32	36	28	4	0
F9 cream	20	52	28	0	0
EA cream	16	44	32	4	4
F9-NLC cream	24	60	16	0	0
EA -NLC cream	20	64	4	12	0

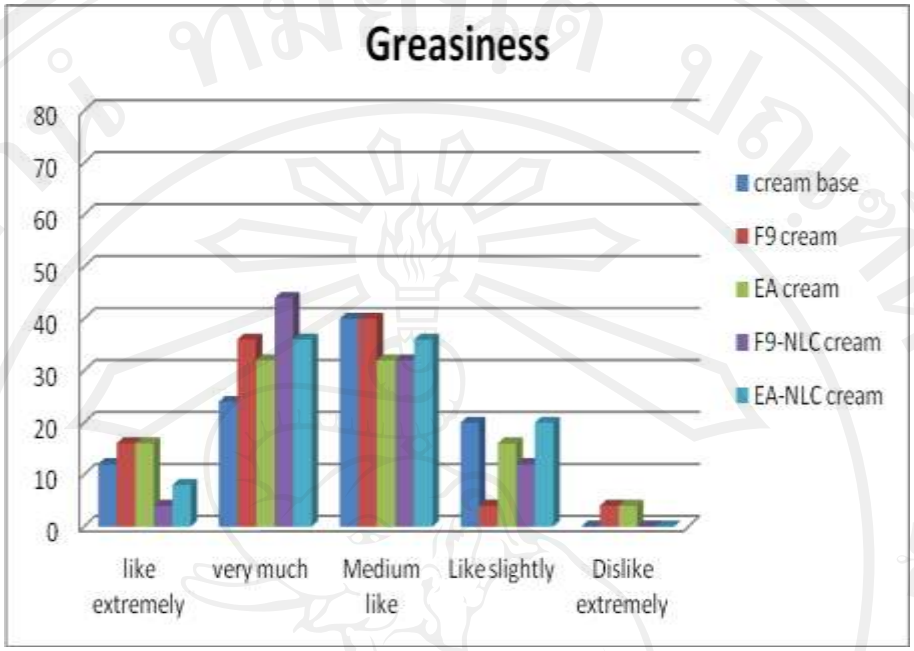
(a)



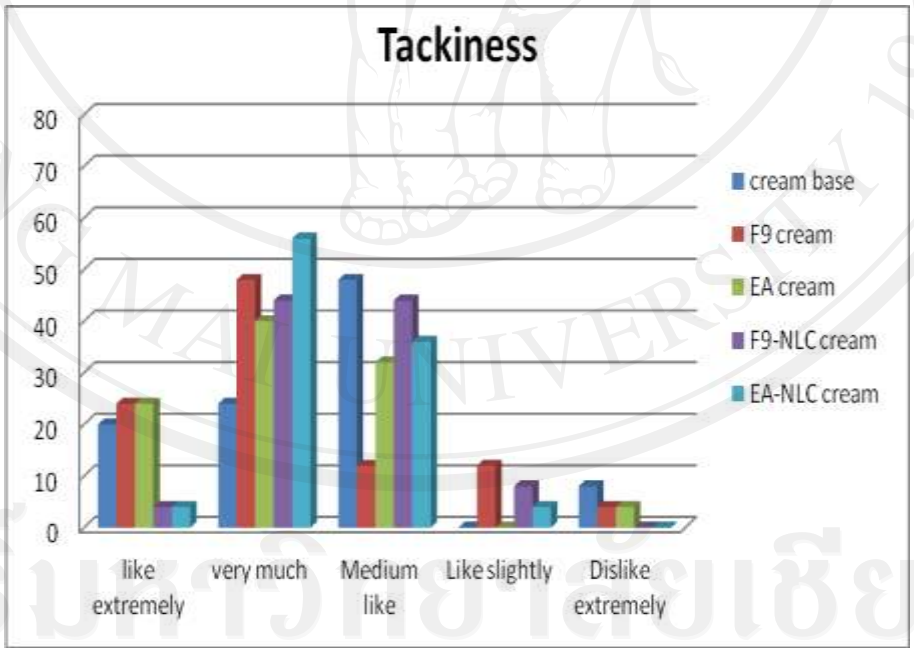
(b)



(c)



(d)



(e)

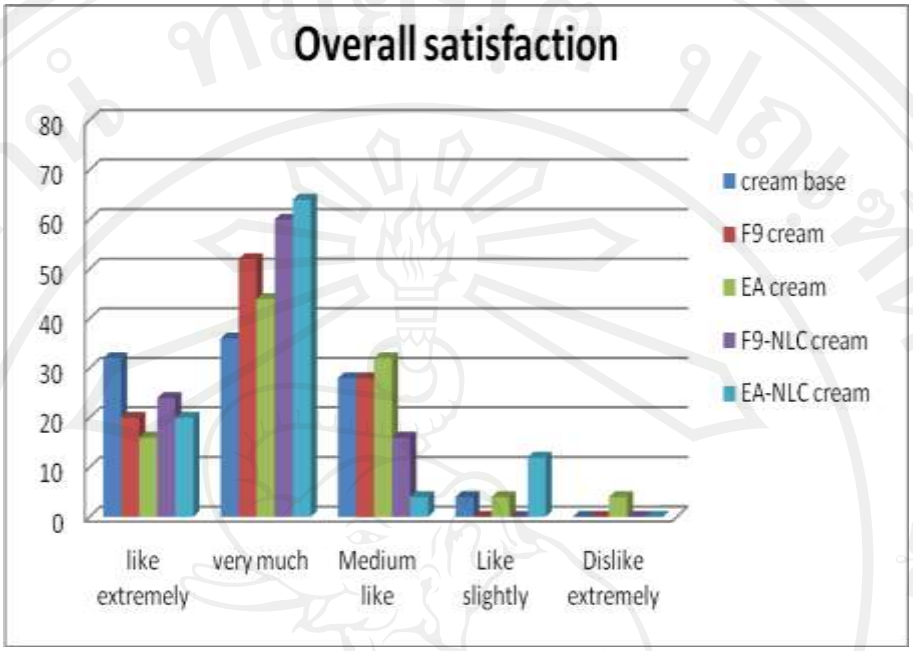


Figure 4.14 The percentage of satisfaction on creams of volunteers**.**